Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 76 APRIL, 1951 NO. 4

SECTION MEETINGS

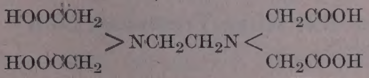
CLEVELAND	
Academy of Medicine	March 9, 1951
ILLINOIS	
Michael Reese Hospital, Chicago	March 6, 1951
MINNESOTA	
University of Minnesota	February 21, 1951
MISSOURI	
Washington University	January 24, 1951
NEW YORK	
New York Academy of Medicine	March 28, 1951

Anti-Coagulant Properties of Ethylene Bis-iminodiacetic Acid. (18577)

FREDERICK PROESCHER.

From the Laboratory, Santa Clara Valley Blood Center, San Jose, Calif.

The ethylene bis-iminodiacetic acid (Synonym: ethylenediamine tetra-acetic acid) a polyaminocarboxylic acid derivative* is one of the most powerful deionizing agents for alkaline earth and metals. It is available as the free acid, and as di-, tri- and tetrasodium salt, under the trade name of Sequestrene. The structural formula of the free acid is as follows:



$$\begin{matrix}HOOCCH_2\\HOOCCH_2\end{matrix}>NCH_2CH_2N<\begin{matrix}CH_2COOH\\CH_2COOH\end{matrix}$$

The free acid is only slightly soluble in water, while the sodium salts are readily soluble. The free acid and its salt do not hydrolyze in aqueous solution, and are stable and effective over a wide range of temperatures and pH.

Dyckerhoff(1), *et al.,* were the first to investigate the anti-coagulant effect of the di-sodium salts of Sequestrene. They found that one half to one mg completely inhibited the coagulation of human and animal blood, and that it was 10 times more effective than sodium citrate. The anti-coagulant effect is due to the binding of the blood calcium ion. It has no effect on blood clotting *in vivo.* The di-sodium Sequestrene has been used in our laboratory for routine blood work for over a year. Because of its powerful anti-coagulant properties, only minimum quantities are necessary. It is less damaging to the cellular elements of the blood, and preserves them longer than sodium citrate and the oxalates. For routine blood examination, a 10% aqueous solution (maximum solubility) is preferable; 0.005-0.01 cc will keep 5 cc of blood from coagulating.

---

* The ethylenediamine tetra-acetic acid was first synthesized by the I. G. Farben Industry in Germany. It is now manufactured in the U.S.A. by the Alrose Chemical Co., Providence, R. I., under the trade name of Sequestrene. Four Sequestrenes are available at present, Sequestrene AA (the free acid), SNA 2 (di-sodium salt), SNA 3 (trisodium salt) and SNA 4 (tetrasodium salt).

1. Dyckerhoff, Marx, and Ludwig., *Zt. f. d. ges. exp. Med.,* 1944, v110, 412.

If blood is kept at 5°C, the red cell and leucocyte counts do not show any significant changes for from 3-5 days. If blood is collected in paraffinized containers, the platelet count remains unchanged for 24 hours; after this time a slight decrease is noted (6%). After the initial drop, there is no appreciable change in the count for about 4 days, as compared with a decrease of about 50% in sodium citrate. Hemoglobin, bilirubin and sedimentation speed remain unchanged for several days, if the blood is kept at 5°C and not shaken excessively. The stainability for the cellular elements remains unchanged for a week or longer.

The agglutinogens of the red cells and the oxydase reaction of granulocytes are not destroyed by Sequestrene NA 2. The red cells are strongly agglutinated by their specific antisera, and the oxydase reaction remains positive after blood has been preserved for 3 weeks at 4-5°C.

When given *per os,* Sequestrene NA 2 is practically atoxic for mammals. Rats fed maximum doses did not show any toxic symptoms except diarrhea. Ninety-six per cent of the administered Sequestrene was excreted unchanged in the feces of rats fed massive single doses. The coagulation time was not altered significantly, and no pathological changes in the cellular elements of the blood were noted.

Dyckerhoff found that rabbits tolerated parenteral injection of as much as 80 mg of ethylenediamine tetra-acetic acid per kg, without toxic symptoms. When 100 mg per kilo were given, the animals developed tetany, but recovered rapidly upon administration of calcium ion. Rabbits tolerated intravenous injections of 40-80 mg per kg daily for 10 days. When given intravenously, Martin(2) found 50 mg toxic for dogs. The animals recovered when injected with 100 mg of calcium gluconate. Sequestrene was well tolerated when injected simultaneously with 100 mg of calcium gluconate.

Preliminary experiments for substituting di-sodium Sequestrene for sodium citrate in preserving human blood, have shown that it preserves blood longer if kept at 4-5°C. 1.5 g di-sodium Sequestrene dissolved in 100 cc of 5% glucose, will keep 400-500 cc of blood from coagulating. The di-sodium salt does not caramelize when sterilized with glucose; the sterilized solution has a pH around 5. Twenty human transfusions with blood preserved in the above solution, did not show any ill effects. No tetany, or changes in the coagulation time were noted. Further transfusion experiments are in progress and will be reported later.

---

2. Martin, G. I., *Meeting A.C.S.,* 1949 Abstracts, 117, 16C.

Received January 3, 1951. P.S.E.B.M., 1951, v76.

Mechanism of Adrenal Involution in the Rat After Treatment with Thiouracil. (18578)

M. X. ZARROW* AND I. G. ZARROW.

From the Biological Laboratories, Harvard University, Cambridge, Mass.

Previous work has shown that prolonged treatment with thiouracil will produce atrophy and damage of the adrenal cortex(1-3). In the rat, this decrease in the weight of the

---

* Present address: Dept. of Biological Sciences, Purdue University, Lafayette, Ind.

1. Bauman, E. J. and Marine, D., *Endocrinology,* 1945, v36, 400.

2. McQuillan, M. T. and Trikojus, V. M., *Brit. J. Exp. Path.,* 1946, v27, 247.

TABLE I. Effect of ACTH (L-41-L) on Weight and Ascorbic Acid of Adrenal Gland.

			Adrenal gland				
						% increase in	
No. of rats	Body wt, g	ACTH, mg	Wt, mg	$\frac{\text{Adrenal wt}}{\text{Body wt, g}} \times 100$	mg % ascorbic acid	Adrenal wt	$\frac{\text{Adrenal wt}}{\text{Body wt}} \times 100$
				Thiouracil			
5	150	—	25.4 ±1.3*	16.9 ±1.6	427	—	—
5	167	6	36.7 ±1.7	21.9 ±0.8	453	44.0	29.5
				Control			
5	189	—	45.1 ±3.1	23.8 ±2.1	450	—	—
5	200	6	63.0 ±4.8	31.5 ±1.8	496	40.1	33.3

* Standard error.

adrenal gland is obvious by the 6th week of treatment with a 0.1% solution of thiouracil in the drinking water(4). If the treatment is continued for a year or more, hemorrhage and necrosis may be observed in the adrenals of such animals(5-7). It is apparent that these changes could result either from a direct action of thiouracil on the adrenal gland or be mediated via the hypophysis cerebri. It has been suggested also that thiouracil affects the adrenal gland indirectly through suppression of thyroid hormone production. The lack of thyroxine could conceivably lead to a myxedematous adrenal(8). The present investigation was undertaken to determine whether the atrophic changes in the adrenal cortex were due in part to a failure in the secretion of ACTH by the pituitary or to a failure of the adrenal gland to respond to ACTH. This was tested by determining the response of the atrophic, adrenal gland of the thiouracil-treated rat to exogenous ACTH.

Material and methods. Fifty-seven male rats of the Harvard-Wistar strain were separated into 4 experimental groups. Two groups of rats were given 0.1% thiouracil in their drinking water starting at 21 days of age and maintained on this regime throughout the entire experiment. The remaining two groups were kept as controls. At 105 days of age, *i.e.* after 12 weeks of treatment with the antithyroidal drug, one group of thiouracil-treated rats and one group of control rats were injected twice daily for 6 days with ACTH.‡ All animals were killed by exsanguination 24 hours after the last injection of ACTH. The adrenal, thyroid and pituitary glands were dissected out and weighed to the nearest tenth of a milligram on a torsion balance. In some of the animals, both the adrenals and pituitaries were analyzed for ascorbic acid by the method of Roe and Keuther(9).

Results. In the first experiment, the rats received a total dose of 6 mg of ACTH in 6 days and were killed 24 hours after the final injection (Table I). The adrenal glands of the control rats weighed 45.1 mg as compared with 63.0 mg for the rats treated only with ACTH, an increase of 40.1%. The animals receiving only thiouracil had adrenals weighing 25.4 mg while the thiouracil-treated rats that were also injected with ACTH possessed adrenals weighing 36.7 mg, an

---

3. Deane, H. W. and Greep, R. P., *Endocrinology,* 1947, v41, 243.
4. Zarrow, M. X. and Money, W. L., *Endocrinology,* 1949, v44, 345.
5. Endicott, K. M., Kornberg, A. and Daft, F. S., *Pub. Health Rep.,* 1944, v59, 49.
6. Glock, G. E., *Nature,* 1945, v158, 518.
7. Dalton, A. J., Morris, H. P., and Dubnick, C. S., *J. Nat. Cancer Inst.* 1945, v5, 415.
8. Statland, H. and Lerman, J., *J. Clin. Endocrinol.,* 1950, v10, 1401.

† Obtained through the courtesy of Dr. Stanton Hardy, Lederle Laboratories, Pearl River, N. Y.

‡ Obtained through the courtesy of Dr. John Mote, Armour Laboratories, Chicago, Ill.

9. Roe, J. H. and Keuther, C. A., *J. Biol. Chem.,* 1943, v147, 399.

TABLE II. Effect of ACTH [58-0(50)] on Adrenal Gland of Thiouracil Treated Rat.

			Adrenal gland			
					% increase in	
No. of rats	Body wt, g	ACTH, mg	Wt, mg	$\frac{\text{Adrenal wt}}{\text{Body wt, g}} \times 100$	Adrenal wt	$\frac{\text{Adrenal wt}}{\text{Body wt}} \times 100$
				Thiouracil		
10	202	—	36.6 ±0.81*	18.5 ±0.67	—	—
5	165	12	46.6 ±3.8	28.2 ±2.3	27.3	48.4
3	182	24	50.3 ±1.6	27.6 ±1.6	30.6	52.4
				Control		
11	223	—	55.4 ±3.9	24.6 ±0.71	—	—
5	194	12	64.6 ±2.6	32.7 ±1.2	17.3	33.7
3	209	24	75.1 ±8.1	35.9 ±4.5	35.0	44.7

* Standard error.

increase of 44%. In spite of the disparity in body weight of the rats due to the depressing effect of thiouracil, comparison of the adrenal weights expressed as mg of adrenal per 100 g of rat gave results comparable to those noted above. The injection of ACTH increased the adrenal weights of the control rats from 23.8 for the uninjected to 31.5 mg % body weight for the injected rats, an increase of 33.3%. The adrenal glands of the thiouracil-treated rats weighed 16.9 mg % body weight while the adrenals of the thiouracil-treated rats injected with ACTH weighed 21.9 mg % body weight, an increase of 29.5%. The ascorbic acid content of the adrenals was 450 mg % for the control rats and 496 mg % for the control rats injected with ACTH. An ascorbic acid content of 427 mg % was found in the adrenal glands of the thiouracil-treated rats and 453 mg % in the adrenal glands of the thiouracil-treated rats injected with ACTH. As could be expected, hyperplasia of the thyroid was observed in the rats receiving thiouracil or thiouracil and ACTH. The thyroids of the normal rats weighed 17.3 mg and after thiouracil treatment 74.6 mg. After injection of ACTH, the normal rats showed an average thyroid weight of 16.7 mg and the thiouracil-treated rats an average thyroid weight of 51.9 mg.

The above experiment was repeated with a second batch of ACTH (Lot No. 58-9[50]) and comparable results were obtained (Table II). The normal, control rats had adrenal glands weighing 55.4 mg, while rats injected with 2 and 4 mg of ACTH had adrenal weights of 64.6 and 75.1 mg respectively. The adrenals of the thiouracil-treated rats weighed 36.6 mg and after injection of 2 and 4 mg of ACTH an increase in adrenal weight to 46.6 and 50.3 mg respectively was obtained.

Discussion. Atrophy of the adrenal cortex after thiouracil treatment and the inability to obtain hypertrophy of the gland following exposure to cold led Zarrow and Money(4) to suggest that the failure was due either to a decreased ACTH production by the pituitary gland or decreased sensitivity of the adrenal gland to ACTH. The second hypothesis was examined in the present investigation and found to be untenable. A comparison has been made of the sensitivity of the adrenal gland of the normal and thiouracil-treated rat to ACTH. The per cent increase in the weight of the adrenal gland was approximately the same for both the normal and thiouracil-treated rats after treatment with ACTH. Thus it would appear that the atrophic, adrenal gland of the thiouracil-treated rat is not only capable of responding to ACTH by an increase in size but has approximately the same degree of sensitivity as the adrenal gland of a normal rat. This would indicate a failure on the part of the pituitary gland to secrete the trophic hormone.

A possible explanation for the failure of the hypophysis to produce ACTH after treatment with thiouracil may reside in the limited numbers and types of cells found in the gland of such animals. It is conceivable that when the pituitary is forced to secrete excessive amounts of TSH, as occurs after thiouracil treatment, it can only do so by a decrease in its production of ACTH. Such an explanation has been offered for the decreased release of TSH and gonadotrophins and increased production of ACTH after stress(10,11). Thus the atrophy of the adrenal gland or the failure to obtain hyperplasia after exposure to cold in thiouracil-treated rats would indicate functional damage to the pituitary part of the pituitary-adrenal axis. It is not to be denied, however, that a decreased amount of ACTH is still being secreted in view of the partial maintenance of the ascorbic acid of the adrenal gland and the depletion of the adrenal-ascorbic acid on exposure to cold(12). This would indicate that greater amounts of ACTH are needed for adrenal hyperplasia than for the adrenal-ascorbic acid depletion. The fact that the pituitary of normal animals can secrete increased amounts of both TSH and ACTH (as seen after exposure to cold) does not invalidate the above hypothesis as the situations are not comparable. In the thiouracil-treated rat the stimulus is such as to bring about an increased secretion of only one of the factors (TSH) resulting in an early appearance of ACTH deficiency. In cold stress the initially increased TSH output eventually decreases as the animal remains under the influence of the stress and as the pituitary secretes greater amounts of ACTH.

Conclusion. 1. Thiouracil produces an involution of the adrenal cortex. 2. The atrophic adrenal gland of the thiouracil-treated rat retains its sensitivity to ACTH. 3. It is suggested that the involution of the adrenal gland of the thiouracil rat is caused by a decreased ACTH secretion by the pituitary gland. This decreased ACTH level may result from the excessive TSH production in the pituitary caused by the thiouracil treatment.

---

10. Selye, H., The Physiology and Pathology of Exposure to Stress, 1950, *Acta, Inc.* Montreal, Canada.

11. Mellgren, J., *Acta Path. Microbiol. Scand.*, 1948, v25, 284.

12. Gabrilove, J. L. and Soffer, L. J., *Endocrinology*, 1950, v47, 461.

Received November 21, 1950. P.S.E.B.M., 1951, v76.

Response of Plasma to Excess of Thromboplastin as a Measure of Prothrombin Activity.* (18579)

L. M. Tocantins, R. R. Holburn, and R. T. Carroll.

From the Department of Medicine, Jefferson Medical College, Philadelphia.

The one-stage method of prothrombin determination proposed by Howell(1) and the two-stage procedure employed by Bordet(2) were used for much important work on prothrombin, but the results obtained with those methods were not regarded as quantitative, since large amounts of prothrombin remained unconverted to thrombin and therefore escaped measurement. Smith and coworkers (3) introduced the two principles which subsequently proved to overcome some of the shortcomings of these two methods. They

---

* Aided by a Grant from the Division of Research Grants and Fellowships in the National Institute of Health.

1. Howell, W. H. and Cekada, E. B., *Am. J. Physiol.*, 1926, v78, 500.

2. Bordet, J. and Delange, L., *Ann. Inst. Pasteur*, 1912, v26, 737.

3. Warner, E. D., Brinkhous, K. M. and Smith, H. P., *Arch. Path.*, 1934, v18, 587.

showed(3) that if plasma was sufficiently diluted and an excess of thromboplastin added, all of the prothrombin could be converted into thrombin, that the resulting clotting time varied inversely as the concentration of prothrombin and therefore could be used as a measure of the amount of prothrombin present. The Smith 2-stage method remains the standard reference method for quantitative work on prothrombin. Quick(4) modified this method by eliminating preliminary dilution of the plasma while retaining the principle of using an excess of thromboplastin. A surprisingly close agreement between this rapid convenient one-stage method and the more complex 2-stage determination was shown often to exist. The well justified popularity of this one-stage method, however, has often caused some workers to overlook its limitations and to extend its use to serious quantitative work on prothrombin. It has been claimed(5) that when an excess of thromboplastin is added to optimally recalcified "undiluted" oxalated plasma, that the clotting time is a function of the prothrombin concentration since, in a system so arranged, prothrombin is the only variable. Much of the confusion in the interpretation of one-stage prothrombin determinations stem from a too sweeping application of this concept. Aside from the fact that much of the prothrombin in the plasma remains unconverted to thrombin during the 12 to 15 second interval required for clotting to occur(6) a variously termed prothrombin convertibility factor (accelerator factor, plasma-kinin, Ac-globulin, factor V or labile factor) (7,8,8a,9,10) forecast by Smith(11) influences the rate of thrombin formation and, therefore, the speed of clotting, independently of the amount of prothrombin present. It appears from the evidence to be presented, that the rate of coagulation of plasma after adding an excess of thromboplastin is further influenced by at least 3 heretofore not sufficiently considered variables: 1. The actual concentration of the plasma in the final clotting mixture. 2. The stability of the plasma being tested. This in itself depends on: (a) the mode of collection of the blood and separation of the plasma, (b) the nature of the contacting surfaces used in the collection, storage and testing of the plasma and (c) its content of stabilizing inhibitors. 3. The source of the thromboplastin employed as activating agent.

Methods. Human blood or plasma was collected with especial precautions using siliconized surfaces throughout for collection, storage, measuring and testing. When an anticoagulant was used, it was placed in the syringe and the blood aspirated directly into it. Four types of plasma were generally employed: (a) Normal, obtained from fasting young men or women; (b) hemophilic, obtained from patients with hereditary hemophilia; (c) post-hemorrhagic, hypercoagulable plasma, drawn from patients soon after a severe hemorrhage not resulting from a systemic disorder of hemostasis; (d) asbestos plasma, *i.e.* normal plasma placed in contact with loose asbestos fibers (10 mg asbestos fibers per 1 ml plasma) for 60 minutes at 20°C, without agitation.

In the calculation of the final percentage concentration of the plasma in any given clotting mixture, any or all of the following variables were taken into account: (a) The per cent plasma volume of the sample of blood (calculated from the hematocrit); (b) the total volume of anticoagulant added to the blood; (c) the total volume of the clotting mixture and the respective volume of each of its ingredients namely, plasma, recalcifying solution, activating agent and diluting fluid. Example: 9 ml of normal blood was aspirated into a syringe containing 1.0 ml of 0.1 M sodium oxalate. The packed cell volume (hematocrit) of this mixture was 30. This meant that the 7 ml of plasma in the sample

---

4. Quick, A. J., *Am. J. Med. Sci.*, 1935, v190, 508.

5. Quick, A. J., *The Hemorrhagic Diseases.* Springfield 1942.

6. Flynn, J. E., Trans. of the Second Josiah Macy, Jr. Foundation Conference on Blood Clotting and Allied Problems, January 1949, v2, 145.

7. Fantl, P. and Nance, M., *Nature*, 1946, v158, 708.

8. Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, v169, 231.

8a. Laki, K., *Schweiz. Med. Woch.*, 1944, v74, 13.

9. Owren, P., *Acta. Med. Scand.*, 1947, suppl. 194.

10. Quick, A. J., *Am. J. Physiol.*, 1943, v140, 212.

11. Smith, H. P., Essays in Biology in honor of H. M. Evans, *Univ. of California Press*, 1943, p 549.

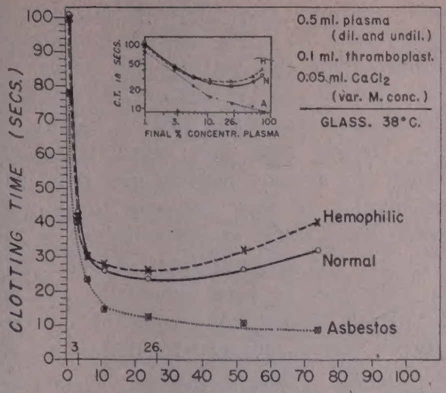


FIG. 1.

Dilution curves of 3 types of plasma activated by a strong thromboplastin. Inset: The same curves plotted on logarithm scale. Asbestos plasma obtained by allowing hemophilic plasma to stand in contact with asbestos fibers, without shaking, for 1 hr at 20°C. (10 mg asbestos fibers, 1 ml plasma). The plasmas were handled by the silicone technic until just before placing them in glass tubes. The concentrations of prothrombin and Ac-globulin of the 3 plasmas tested by the two-stage method(12) were within normal limits. Plasmas obtained from citrated blood (0.2 ml 19% citrate to 10 ml of blood, hematocrit 38). Dilution of the plasma in the first clotting mixture 60/62 × 50/65 or a starting plasma concentration of 74%.

was diluted by 1 ml of the anticoagulant (or a dilution of 6/7). In preparing the initial clotting mixture, 0.5 ml of this already diluted plasma was added to 0.05 ml of thromboplastin followed at once by 0.05 ml 0.2 M $CaCl_2$, thus involving a futher 5/6 dilution of the plasma. Therefore 6/7 x 5/6 x 100 = 71% final plasma concentration in the first clotting mixture. By using 19 or 38% citrate solution as anticoagulant and increasing the volume of plasma in the clotting mixtures, it is possible to work with higher plasma concentrations.

Results. 1. *Relation of the actual concentration of the plasma in the final clotting mixture to action of thromboplastin.* If a strong solution of an aqueous extract of human brain is added to normal, hemophilic and asbestos plamas, beginning with a concentration of plasma in the actual clotting mixture of 74% and progressing down to one per cent, curves such as those illustrated in Fig. 1 are the result. The plasmas used in this test were not allowed to come into contact with glass until the actual testing. Without these precautions the results are erratic. The greatest differences between the three types of plasma are in the clotting mixtures of high plasma concentration. High plasma concentrations slow the action of thromboplastin. Even slight dilution shortens the clotting time of hemophilic and normal plasma; little change in their rate of clotting is observable in the mixtures of plasma concentration between 10 and 30%. Asbestos plasma from which much of the action of first phase inhibitors is removed, is hypercoagulable even in high contentrations, and its clotting time gradually lengthens from the start, as the plasma is diluted. Whether on simple arithmetic or logarithmic ruled charts, (Fig. 1) the curves for hemophilic and normal plasma follow a parabolic course; the curve for asbestos plasma follows a hyperbolic course when plotted in arithmetic ruling, but rectilinear in logarithmic ruled paper. Asbestos plasma resembles in many of its characteristics, plasma that has lost much of its stability after being collected and stored in glass tubes.

The total volume of each of the clotting mixtures represented in Fig. 1 is, however, 0.65 ml. Let us see what results when the volume of the mixture is 0.3 ml (as in the one-stage method); the thromboplastin is kept constant and the amount of calcium varied to be optimal for concentrations of normal plasma from 70 to 4% (Fig. 2, Curve A). The clotting times observed may then be compared with those obtained by diluting the plasma in the conventional Quick one-stage procedure, again using the same total volume, amount of thromboplastin and optimal recalcification (Fig. 2, Curve B). When the activity of dilutions of plasma tested by the one-stage "prothrombin time" method is plotted with due attention to the final per cent plasma concentration in each clotting mixture, the curve (Curve B) almost superimposes a segment of curve A, which had its origin at high plasma concentration (70%). Curve B, therefore, when correctly plotted, is only a small part of Curve A, mainly its ascending segment; it begins at 26 to 28%

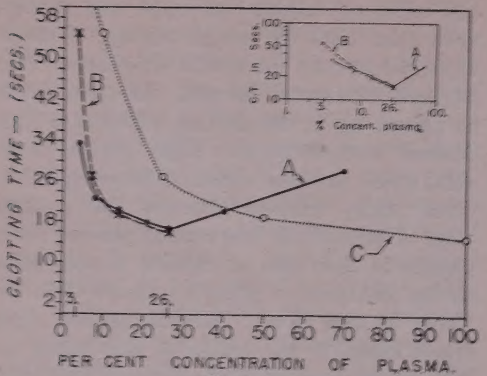


FIG. 2.

Curve A: The effect of adding a strong thromboplastin to stable normal plasma, at different concentrations of the plasma in the clotting mixture. Total volume of the clotting mixture: 0.3 ml throughout. Silicone technic used up to the point when the plasmas were placed in the glass clotting tubes. Plasmas derived from oxalated human blood (9 ml blood plus 1 ml sodium oxalate). Concentration of the thromboplastin the same throughout. *Curve B*: Curve plotted from values obtained by testing the same plasma, according to the Quick one-stage "prothrombin time" method. Total volume of the mixture 0.3 ml. Same concentration of thromboplastin as that used for values in curve A. The points on this curve as well as on curve A represent the final concentration of the plasma in the actual clotting mixture. *Curve C*: The points on curve C are the same as those of curve B, but are plotted according to the common practice of referring plasma concentration not to the actual clotting mixture, but to the alleged original concentration of the plasma without regard for dilution by the anticoagulant and the other clotting reagents. It is the familiar dilution curve used for calculation of "prothrombin concentration" by the one-stage method.

plasma concentration or at the bottom of the valley of Curve A. It can now be seen how far these two curves differ from Curve C, said to express the relation between concentration of plasma (or of prothrombin) and activated clotting time in seconds(5). The one-stage method as currently performed covers therefore the response of plasma to thromboplastin only from plasma concentrations of about 26% or below.

2. *Response of plasmas of variable stability (normal, hemophilic, posthemorrhagic) to addition of strong thromboplastin.* Various authors have stated that hemophilic plasma does not differ from normal in its response to a strong thromboplastin solution. Though such might be the case with unusually strong thromboplastins (*e.g.* placental extracts) the usually reported lack of difference in response between these two plasmas can result from tests done in clotting mixtures in which the plasma concentration is 26% or below. We have already seen how much hemophilic, normal and asbestos plasmas may differ from one another in their response to thromboplastin when tested at concentations of 74% (Fig. 1). At concentrations of about 26% the differences between hemophilic and normal plasma become non-significant, but those between normal and asbestos plasma are still substantial. When a plasma concentration of about 3% is reached (that used in the Link-Shapiro method) the differences between the three plasmas become correspondingly less.

In Fig. 3 are represented the response of 3 types of plasma to the same amounts of thromboplastin, employing 2 clotting mixtures of different plasma concentration. The hypercoagulable plasma was obtained from a pa-

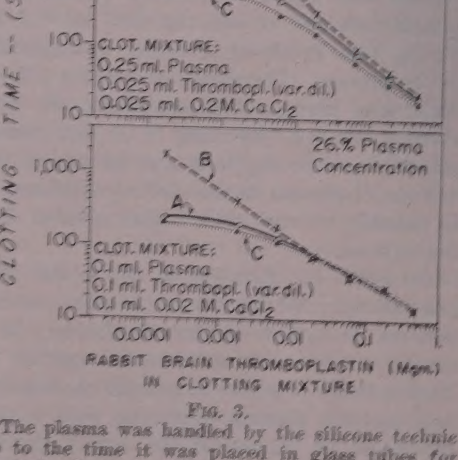


FIG. 3.

The plasma was handled by the silicone technic up to the time it was placed in glass tubes for testing. Rabbit brain thromboplastin prepared from acetone dried rabbit brain. Blood collected by the silicone technic (9 ml blood plus 1 ml 0.1 M sod. oxalate).

tient after hemorrhage and transfusion. The 3 plasmas had a normal content of prothrombin and Ac-globulin (2-stage method)(12). As shown in the chart, the clotting times were all shorter at 26 than at 70% plasma concentration, even though the latter clotting mixture had more prothrombin and Ac-globulin, and both received the same amount of thromboplastin. While no difference was demonstrable between the clotting times of the 3 plasmas at 26% plasma concentration when 0.02 mg or more thromboplastin were used, a significant difference was evident when the plasmas were tested at 70% plasma concentration. In the presence of amounts of thromboplastin (0.0018, 0.00018 mg) which easily clotted hemophilic plasma in a mixture of 26% plasma concentration, the same plasma remained incoagulable when tested in 70% concentration. Likewise at the higher plasma concentrations, differences were manifest between the normal and hypercoagulable plasma that were small or nonexistent in the 26% plasma.

3. *The rate of coagulation of stable normal plasma at various concentrations with or without the addition of an activating agent.* Citrated plasma obtained from a normal man was diluted with 0.85% NaCl in such a way that its concentration in the clotting mixture varied between 90 and 1.8%. In one series of mixtures no activating agent was added (non-activated plasma). In another series, an activating agent (thromboplastin) of uniform concentration was included in the clotting mixture (activated plasma). The results are represented in Fig. 4. Dilution of the non-activated plasma accelerated coagulation even when the plasma concentration in the clotting mixture was between 1 and 2%. When the plasma was activated by an aqueous extract of human brain, the greatest acceleration of coagulation was detected at plasma concentrations between 20 and 40%. The addition of thromboplastin depressed the curve at its descending limb, representing clotting times of mixtures of high plasma concentration (90 to 40%). It is conceivable that, if even greater increments of thromboplastin could be added to the mixture, this section of the curve could be further depressed, until it might assume a rectilinear or at least a hyperbolic course (like that of activated asbestos plasma). This was tried by adding highly concentrated brain extract, starting with a dried lyophilized aqueous brain extract. It was not possible, however, to have sufficient thromboplastin activity to accelerate the clotting time of the mixture with highest plasma concentration to the point that it would clot faster than the more dilute plasma mixtures; provided, of course, the plasma being used was stable, and had been collected and processed on silicone surfaces.

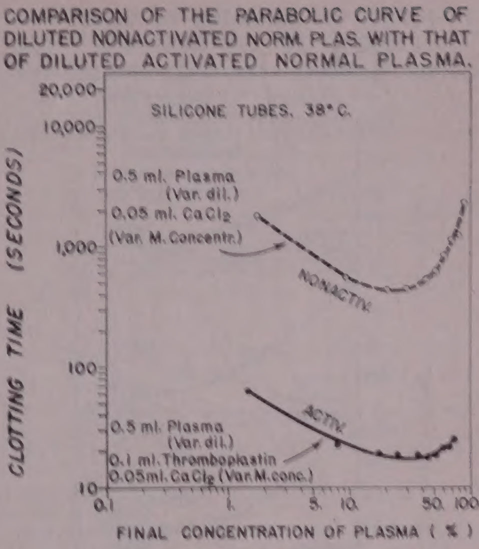


Fig. 4.

Stable plasma collected with the silicone technic and tested on silicone surfaces. 0.85% NaCl used in diluting plasma. Thromboplastin: aqueous human brain extract.

If the plasmas have been allowed to be in contact with glass surfaces, their clotting times become progressively shorter (Fig. 5). After 6 days exposure to glass there is no significant difference between the response of hemophilic and normal plasmas to thromboplastin. The dilution curves lose their parabolic character and the mixture of highest plasma concentration has the shortest clotting time.

---

12. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

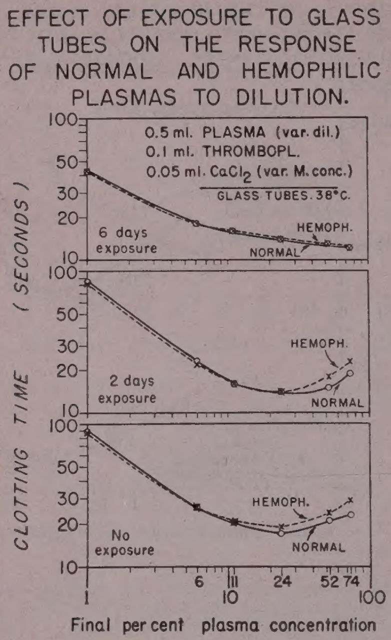


Fig. 5.

Plasmas collected with the silicone technic, then stored at 5°C after testing, in glass tubes for the periods shown.

In Table I are grouped the results of testing the prothrombin activity of 4 plasmas of varying stability using methods employing clotting mixtures from 0.5 to 70% plasma concentration. Prothrombin activity of the plasmas as measured by the 2-stage method does not differ significantly; when tested by one-stage methods at relatively higher plasma concentrations, divergent results follow. When the plasmas are tested at 70% concentration they either resist (*e.g.* hemophilic plasma) or yield too readily (asbestos plasma) to activation by thromboplastin, in either case failing to reflect quantitatively the amount of prothrombin in each plasma.

4. *Response of plasma to heterologous thromboplastins.* If dilute solutions of Russell Viper Venom are used as an activating agent, the clot accelerating effect of moderate dilution of the plasma is not observed with either normal or hemophilic plasma(13). Furthermore, regardless of how high the concentrations of plasma may be in the mixture, there is no significant difference between the response of hemophilic and normal plasma to the venom until small amounts of the venom are used. Attention was drawn previously to the fact that Russell viper venom was not vulnerable to the lipid anticephalin extracted from blood or plasma(14) and that this may underlie the lack of agreement between estimations of prothrombin activity by the one-stage method when using rabbit or human brain thromboplastin as against Russell Viper Venom(15). On Table II is shown the ineffectiveness of the lipid anticephalin extracted from human brain in a clotting system activated by Russell Viper Venom, in contrast with one activated by human brain thromboplastin. The invulnerability of the venom to the natural first phase inhibitor may explain why at low prothrombin levels, as those induced by dicumarol, the clotting time of plasma activated by brain or lung thromboplastin is proportionately longer than when activated by Russell Viper Venom. A species preferential action has been demonstrated for thromboplastin(5) as well as the plasma and tissue antithromboplastin(14,16,17). It seems important therefore, that, in studies on rates of prothrombin conversion or utilization, homologous thromboplastin be employed. Without this precaution, the full extent of a defect in prothrombin conversion may not be appreciated.

Discussion. A distinction should obviously be made between the final concentration of the plasma in the actual clotting mixture, and the original concentration of the plasma before it is added to the other clotting reagents. The one-stage methods refer to the original percent concentration of the plasma. Likewise, the familiar hyperbolic activity curves for various

---

13. Tocantins, L. M., *Am. J. Physiol.*, 1945, v143, 67.

14. Tocantins, L. M., Carroll, R. T., and McBride, T. J., Proc. Soc. Exp. Biol. and Med., 1948, v68, 110.

15. Tocantins, L. M., Proc. Soc. Exp. Biol. and Med., 1944, v55, 291.

16. Tocantins, L. M., *Am. J. Physiol.*, 1943, v139, 265.

17. Fantl, P. and Nance, M. H., *Med. J. Australia*, 1947, v2, 125.

TABLE I. Results of Testing 4 Plasmas for Prothrombin Activity Using Clotting Mixtures of Various Plasma Concentration. Silicone technic for collection and storage of plasma. Clotting tested in glass tubes at 38°C.

	Prothrombin activity							
	One-stage method						Two-stage method§	
	Quick*		Link *et al.*†		Present paper‡			
Type of plasma	Time (sec.)	% of normal	Time (sec.)	% of normal	Time (sec.)	% of normal	Units per ml plasma	% of normal‖
Normal	13	100	41	100	16	100	345	111
Hereditary hemophilia	13	100	41	100	21	<100	325	104
Normal + lipid antithromboplastin (0.04 g %)	16	52	43	<100	25	<100	360	116
Asbestos plasma¶	10	>100	40	100	10	>100	375	120
Actual % conc. of plasma used in clotting mixture	26		3		70		0.4	

* 0.1 ml plasma, 0.1 ml thromboplastin, 0.1 ml 0.02 M $CaCl_2$ (PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 788).

† 0.1 ml 12.5% plasma, 0.1 ml thromboplastin, 0.1 ml 0.025 M $CaCl_2$ (*J. Biol. Chem.*, 1941, v138, 1).

‡ 0.25 ml plasma, 0.025 ml thromboplastin, 0.025 ml 0.2 M $CaCl_2$.

§ Warner, Brinkhous, and Smith method as described in Ware and Seegers, *Am. J. Clin. Path.*, 1949, v19, 471.

‖ Compared with activity of frozen control normal plasma (310 units prothrombin per ml plasma).

¶ Normal plasma in contact with asbestos fibers for one hour at 20°C (10 mg asbestos per ml plasma) (Tocantins, *Am. J. Physiol.*, 1945, v143, 67).

TABLE II. Comparison of Action of Brain Lipid Anticephalin Against Two Types of Thromboplastic Agents.

	Lipid anticephalin (mg in clotting mixture)							
Activating agent	1	0.5	0.2	0.1	0.05	0.02	0.01	0.85% NaCl
	Clotting time in seconds							
Russell viper venom*	18	18	19	19	20	20	20	20
Aqueous human brain extr.†	310	247	195	128	85	49	30	15

* 0.1 ml venom solution, 0.1 ml lipid anticephalin (or 0.85% NaCl), 0.1 ml plasma, 0.1 ml 0.02 M $CaCl_2$.

† 0.1 ml brain extract, 0.1 ml lipid anticephalin (or 0.85% NaCl), 0.1 ml plasma, 0.1 ml 0.02 M $CaCl_2$.

dilutions of plasma activated by a strong thromboplastin refer to the original plasma concentration and not to that in the final clotting mixture. The greater the actual concentration of the plasma, the more it resists the action of an excess of thromboplastin. The more careful and thorough the collection and separation of the plasma, the greater is its antithromboplastin activity(16). Skillful venepunctures and protection of the blood and plasma by using silicone coated surfaces, enables the plasma to retain its property of resisting the action of thromboplastin. Such precautions are seldom taken (or indeed necessary) for the determination of prothrombin activity by the one-stage method. They are, however, along with the maintenance of a high plasma concentration in the clotting mixture, essential for the demonstration of the true response of the plasma to activation by thromboplastin.

It appears that only in sufficiently diluted plasma, can the response to an excess of thromboplastin be taken as an indication of the amount of prothrombin present. In undiluted or slightly diluted plasmas, the action of thromboplastin is resisted, prothrombin conversion is correspondingly delayed and the response of the plasma can no longer be depended upon as an indication of the amount of prothrombin present.

A clotting time of 12 to 15 seconds results when oxalated (or citrated) normal human plasma is added to equal volumes of thromboplastin and calcium, and is said to be equivalent to 100% prothrombin activity. This does not mean, however, that the prothrombin concentration in such a clotting mixture is 100%, since it is obvious that the test itself involves diluting the plasma threefold, and this, added to the dilution introduced by the anticoagulant, gives a clotting mixture with an actual plasma concentration of about 26%. But, since both unknown and normal control plasmas are similarly diluted, it is proper to express the findings in the unknown plasma in terms of per cent of activity of the control, but incorrect to speak of them as expressing concentration of prothrombin. Prothrombin activity as currently measured is a function not only of the amount of prothrombin present but also of accompanying inhibitors and accelerators which, directly or indirectly, affect its conversion rate. Heretofore, only platelets, plasma and serum accelerators have been generally recognized to influence prothrombin conversion rate. The role of natural inhibitors in the plasma was poorly appreciated principally because, as shown above, these inhibitors were placed at a disadvantage by the use of diluted plasmas and strong heterologous thromboplastins, in tests for prothrombin activity. It may be assumed that much information may be gathered regarding the role of these inhibitors in regulating prothrombin conversion, by utilizing clotting systems with high plasma concentration, suitable contacting surfaces and moderately strong homologous thromboplastins as activating agents(18).

Summary. 1. The response of varying concentrations of stable normal plasma to strong homologous thromboplastin may be expressed by a curve of the parabolic type. Clotting mixtures of high concentration resist activation by thromboplastin, an effect which is more evident with abnormally stable plasmas like those from hemophilics. 2. All current one-stage methods for determining prothrombin activity are done on diluted plasma. The actual plasma concentration in the Quick one-stage procedure is about 26%. 3. In clotting mixtures with a high plasma concentration the action of thromboplastin is resisted, prothrombin conversion is delayed and the rate of clotting of such plasmas cannot be used as an indication of the amount of prothrombin present. Final plasma concentrations of 5% or under in the actual clotting mixture are necessary to be able to derive dependable correlations between the rate of clotting and prothrombin activity. 4. For a *full* demonstration of plasma anticephalin (antithromboplastin) activity, clotting mixtures with a high plasma concentration, a moderately strong homologous thromboplastin and inert contacting surfaces (*e.g.* silicone) are essential.

18. Tocantins, L. M., Carroll, R. T., and Holburn, R. R., Trans. Third Josiah Macy, Jr. Foundation Conference on Blood Clotting, 1950, in press.

Received January 9, 1951. P.S.E.B.M., 1951, v76.

Feeding Tests with Some Algin Products. (18580)

HUGO W. NILSON AND JOHN A. WAGNER.

From the Fishery Technological Laboratory U. S. Fish & Wildlife Service, College Park, and the Department of Pathology, University of Maryland School of Medicine.

Algin products are widely used in food and pharmaceutical products as stabilizing, thickening, suspending, emulsifying and gel producing agents, because of their pronounced hydrophilic, colloidal properties. They are derivatives of alginic acid which is a polymer of anhydro-β-D-mannuronic acid units extracted from the giant kelp *(Macrocystis pyrifera)*. Three algin products manufactured by the Kelco Co. of San Diego, Calif.

covered here are: (a) Dariloid, a sodium alginate standardized to uniform colloidal strength with sugar and dextrin, and treated with phosphate to make it soluble in milk. It is used as a stabilizer in ice cream and other food products. (b) Kelgin, a sodium alginate used in foods as a stabilizer, thickener or gel-forming agent and in pharmaceuticals. (c) Kelcoloid, a propylene glycol alginate used as an emulsifier, bodying agent and stabilizer in foods such as French dressing, and in pharmaceuticals. These algin products are used in aqueous solution at levels of about 0.1 to 0.5% by weight of the finished product.

The feeding studies were conducted under a cooperative agreement with the manufacturer in order that the U. S. Fish & Wildlife Service might obtain data on the nutritive value of seaweed products and be better able to recommend a rational system of utilization of this important natural resource. The products used were purchased on the open market.

Experiments with rats. 10-week comparable feeding tests begun in March, 1939 have been reported for algin (Dariloid) and gelatin(1), and for agar and Irish moss(2). In these experiments the levels of seaweed preparations fed were 5, 10, 20 and 30% by weight of the diet. Similar 10-week feeding experiments were conducted during that time with Kelgin which have not been reported previously. The individual gains in weight, and food and water consumption were quite similar to those of rats fed Dariloid. The mean daily gains of the groups of rats fed the 5, 10, 20 and 30% levels of Kelgin for the 10-week period were 3.81, 3.47, 2.91 and 2.35 g respectively. The mean total food consumed per g gain in weight was 3.20, 3.26, 3.48 and 3.87 g respectively. The mean water consumed per g gain in weight was 5.84, 6.28, 8.83 and 13.74 g respectively. Only 2 instead of 4 out of 6 rats per group survived on the 2 higher levels of Kelgin. The deaths of these 8 rats during the first 2 weeks were apparently not due to Kelgin *per se* but indirectly to the low absorption quality of the basal diet. Long duration experiments were limited for both Dariloid and Kelgin to diets containing the 5% level. Only gross post-mortem studies were done since the animals were permitted to die naturally. Experiments, both short and long duration, with Kelcoloid fed at various levels in the diet were begun in July, 1946. Male albino rats (Yokelson's strain) were used in the experiments with Dariloid and Kelgin. Male and female albino and hooded rats (College Park laboratory strains) were used in the experiments with Kelcoloid. Rats weighing about 50 g and about 4 weeks in age were allotted into groups of about 10. They were individually caged and were offered food and water free-choice. The live weight, and food and water consumption data were collected weekly. A uniform environmental temperature of 80°F was maintained. The diets for rats, except in one instance, consisted of algin at the desired level; casein, 15; lactalbumin, 5; lard, 15; dried brewer's yeast, 5; wheat germ, 2; cod liver oil, 2; salt mixture (U.S.P. XII, No. 2 for vitamin A or D assay) 4, and dextrin and sucrose in about equal quantities to make a total of 100 parts by weight. After the forty-fifth week, 0.5% dried liver extract, Lilly, was added to the diet containing Kelcoloid to determine if additional vitamins might effect a change in texture of feces. No change was noted. A single group of rats was fed the 15% level of Kelcoloid in ground Purina Dog Chow.

The data in Table I indicate no significant effect of the 5% level of algin on longevity. The data in Table II indicate that the group fed the 5% level of algin had similar mean maximum weights and consumed similar quantities of food and water as the controls. The higher levels of Kelcoloid did reduce life expectancy somewhat but not as much as might be expected from the comparatively large intake of food and water. It is surprising how well the rats fed the 15 and 25% levels of Kelcoloid were able to handle the bulky diet which caused loose and smeary feces. The rats fed the 15% level of Kelcoloid in the Purina Dog Chow diet grew very well during

---

1. Nilson, H. W. and Lemon, J. W., U. S. Dept. Int., *Fish and Wildlife Service, Res. Rpt.*, 1942, No. 4.

2. Nilson, H. W. and Schaller, J. W., *Food Res*, 1941, v6, 641.

TABLE I. Length of Life in Weeks of Rats Fed Different Levels of Algin.

Diet designation	Length of life in weeks											
Control	53	66	83	90	93	95	98	101	104	123	129	132
Dariloid: 5%	30	62	64	67	76	80	89	96	99	128		
Kelgin: 5%	45	67	70	72	83	85	90	93	101	128		
Control	5	14	63	*64*	*73*	75	*76*	*85*	*94*	109		
Kelcoloid: 5%	*10*	*10*	21	37	*41*	*62*	75	76	94	*104*		
15%	1	*1*	*2*	3	*3*	*43*	66	70	*78*	*79*		
15%*	25	36	†									
25%	5	*13*	15	*44*	*57*	*57*	*58*	*66*	79	86		

Italicized data are for female rats.
* These rats were fed the Purina Dog Chow diet.
† Others sacrificed at about 37 wk.

TABLE II. Data on Feeding Tests with Algin Conducted from Weaning to Death of Rats, Except as Noted.

Diet designation	No. of rats	Mean max. wt, g	Mean weekly algin intake, g	Mean weekly basal diet intake, g	Coef. of var. of food intake, %	Mean weekly water intake, ml	Coef. of var. of water intake, %
Control	12	578	0.0	92.2	9	159	19
Dariloid: 5%	10	567	4.5	86.6	14	165	16
Kelgin: 5%	10	577	4.6	88.6	9	168	15
Control	10	292	0.0	61.9	18	130	25
Kelcoloid: 5%	10	292	3.3	63.0	17	149	25
15%*	5	245	10.3	58.5	20	169	21
15%†	10	251	13.9	78.5	14	225	12
25%	10	130	11.3	33.7	37	129	40

* Only rats which lived for at least 3 wk are included.
† These rats were fed the Purina Dog Chow diet, and 8 survivors were sacrificed after 37 wk.

the 37-week period, and the feces were normal in consistency. The early deaths of the rats fed the purified diet containing 20 and 30% Kelgin and 15% Kelcoloid must have been due to inanition which was indicated in the food consumption records. The apparent digestibility of the algin content of Dariloid during the tenth week was 3% for rats fed the 5% level, 36 for the 10, 63 for the 20 and 88% for the 30% level. The amount digested seemed to depend on the concentration in the diet, and may have been dependent on the changed bacterial flora in the intestines. For Kelgin it was none for rats fed the 5% level, 67 for the 10, 76 for the 20 and 78% for the 30% level. For Kelcoloid, 8 out of 23 rats apparently did not digest any algin. The other 15 showed a mean apparent digestibility of 15% irrespective of the level fed. The range in apparent digestibility for individual rats was 0.4 to 34%. The lowest level of algin fed to the rats represents a minimum of 10 and a maximum of 50 times the quantity of algin used in a foodstuff or pharmaceutical product for human use. The rats also had to consume this level in their entire food intake while algin is seldom consumed in more than a single item in the daily diet of humans. The data on longevity, maximum weight, and food and water consumption indicate no adverse trend that can be ascribed to the algin *per se* (Tables I and II). The higher mortality rate, decreased size, etc., associated with the higher levels of algin can be ascribed to the greater bulk, and water absorbing capacity of the colloidal material which seemed to limit the intake of nutrients. There was no differential effect on the two sexes.

In the experiments with Dariloid and Kelgin, the gross necropsy studies showed no recurring symptoms which indicated a toxic condition caused by the algin. In the experiments with Kelcoloid microscopic anatomy studies for all animals were made of the brain, heart, lung, stomach, liver, spleen, kidney, small intestines, large intestines, and ovaries or testes whenever an animal was sacrificed or, if found dead, before decomposition had advanced too far. As many rats as possible which died during the later part of the experiment were examined. Death of rats fed control and Kelcoloid diets was usually due to myocardial fibrosis, pneumonia and the multiplicity of cumulative processes associated with aging. There were no lesions which could be attributed to a toxemia or to a local irritative intestinal process. Renal calcareous deposits were found frequently in animals that died during the latter part of the experiment in control as well as experimental animals and probably were related to some extraneous dietary constituent. In summary, algin is a wholesome food product as indicated by these feeding tests with rats.

Experiments with mice. Mice weighing 12 to 18 g were allotted into 4 groups which were fed Ground Purina Dog Chow containing 0, 5, 15 and 25% by weight of Kelcoloid, respectively, for one year. The mice were caged individually. None of the mice in the control group died naturally during the year. Mice died during the following weeks in the other groups: 5% level, 13, 48, 48 and 50; 15% level, 13, 13 and 31, and the 25% level, 1, 13, 13, 20 (escaped), 36, 39, 45 and 48. One mouse in the control group, 1 fed the 15% level and 2 fed the 25% level were sacrificed after 39 weeks. The others were sacrificed after a year. The mean maximum weights were 34.5, 34.8, 31.6 and 28.6 g respectively for mice fed the 0, 5, 15 and 25% levels of Kelcoloid. The mean weekly consumption of Kelcoloid, Purina Dog Chow and water was 0 and 31.6 g and 13.1 ml for mice in the control group; 1.7 and 32.5 g and 12.0 ml for mice fed the 5% level; 5.0 and 28.3 g and 13.5 ml for mice fed the 15% level, and 7.9 and 23.8 g and 16.5 ml for mice fed the 25% level of Kelcoloid. There were no outward symptoms of toxicity. No lesions were found during a study of the microscopic anatomy of important organs which could be definitely attributed to any single process. There was, however, a higher mortality rate and smaller mean maximum weight of mice fed the 2 higher levels of Kelcoloid. This apparently was due to the water absorption quality of the diet which caused enough bulk to limit the intake of nutrients.

Experiments with guinea pigs. The pigs were fed Champion Mink Food containing 0, 5, 10 and 15% Kelcoloid respectively. After 2 control pigs and 2 fed the 5% Kelcoloid level died within 8 weeks, the diet was changed to include 50% of an equal mixture of ground yellow corn and standard wheat middlings. In addition 10 ml of tomato juice and a limited quantity of poor grade timothy hay was fed. After 26 weeks the control pigs had final weights of 642, 648 and 708 g and a mean weekly intake of concentrate feed of 155 g per pig. Two of these pigs were replacements after the seventh week. The single pig remaining in the group fed the 5% level of Kelcoloid had a final weight of 627 g and had a mean weekly intake of 146 g of concentrate feed. The pigs fed the 10% level of Kelcoloid had final weights of 557, 585 and 688 g and a mean weekly intake of concentrate feed of 152 g per pig. The pigs fed the 15% level of Kelcoloid had final weights of 570, 602 and 633 g and a mean weekly intake of concentrate feed of 157 g per pig. The study of microscopic anatomy of organs showed no lesions except mild cloudy swellings of the liver and kidneys in any of the control or Kelcoloid pigs. In summary, all pigs grew well after the diet had been altered to include more vitamin C, carbohydrates and roughage. The Kelcoloid was without untoward effect at any level fed in the diet.

Experiments with chicks. Day-old New Hampshire chicks were allotted to 4 groups and fed Purina Starting Mash containing different levels of Kelcoloid. At first the selected levels were 0, 10, 25 and 50% Kelcoloid. During the first day it became apparent that the chicks receiving the 25 and 50% levels could not eat the diet. It oftentimes formed large masses on the beaks. The chicks fed these levels for 1 day were then fed the control diet for 1 week. At the close of this period they were fed diets containing 5 and 15% Kelcoloid. The data in Table III show that the Kelcoloid even in the lowest level apparently reduced the growth rate although these chicks ate about the same as the control chicks. The Kelcoloid may have inter-

TABLE III. Data on Feeding Studies with Chicks.

Diet designation	No. of weeks	No. of chicks Initial	No. of chicks Close	Mean wt of survivors, g	Mean weekly intake Kelcoloid, g	Mean weekly intake Basal diet, g
Control	7	13	11	602	0	189
Kelcoloid: 5%	7	13	12	505	10	195
10	7	12	6	316	15	130
15	3	13	4*			

* Survivors were killed after 3 wk for study of microscopic anatomy.

TABLE IV. Data on Feeding Tests with Cats.

Identification	Duration, days	Weight Initial, g	Weight Final, g	Mean daily intake Kelcoloid, g	Mean daily intake Pard, g	Mean daily intake Salmon, g
LMR	14	2335	2144	6.4	40.7	32.3
LR	14	2475	1973	15.8	68.3	37.9
1*	88	2030	975	7.4	66.2	22.1
2	110	2077	2007	5.0	91.2	27.8
3	57	2273	2125	0.0	95.6	33.4
	54	2125	1735	8.9	80.4	26.8
4†	45	2031	1152	5.4	89.0	32.4
5	90	2192	2197	0.0	99.4	29.8
6‡	33	1960	1250	14.6	72.4	31.6
8	90	2050	1004	6.0	54.2	16.3
Mother cat		2421	2742			
Kitten		542	1485			

* This cat did not eat well towards latter part of period. It seemed strong in the evening, but died during the night.

† This cat seemed suffering from a bad cold for 2 days before it died. The eyes were swabbed with boric acid solution, and 20,000 units of penicillin were given intraperitoneally.

‡ This cat was extremely emaciated and practically moribund, so it was sacrificed.

fered with the utilization of nutrients. In the 2 higher levels there was evidence that the physical condition of the diet limited food intake sufficiently so that the chicks could not survive or grew very poorly. It was almost a physical impossibility for the chicks to eat the diet containing the 15% level of Kelcoloid. A variety of lesions of minor importance was seen in the organs at irregular and insignificant intervals. Although the chicks had not survived or grown well in many instances, it was concluded from this study that the anatomical lesions indicated slight evidence of transient reversible tissue changes in both control and Kelcoloid chicks. There was no evidence of chronic toxicity.

Experiments with cats. The cats were fed a diet of canned Pard dog food and canned salmon for at least a month until there were no gross symptoms of disease or parasites. One cat had a litter of kittens of which 1 survived. The mother cat and kitten were fed the same diet and were used as a negative control at the close of the experiment. Two cats were sacrificed after a 2-week feeding test. The remainder of those that survived were fed from 88 to 111 days and were sacrificed. The quantity of diet offered to all cats except the mother cat and kitten was limited to 100 g of Pard diet containing 0, 5, 10 or 15% Kelcoloid, and 30 g of canned salmon. None of the cats fed Kelcoloid would consume any more than this quantity of food. Cat 3, during the control period, and cat 5 barely maintained their weight on this quantity of food. It is not surprising that the other cats lost weight (Table IV). The cats had trouble eating the diet containing Kelcoloid seemingly because of the physical texture. They had difficulty in swallowing, also. The cats which received the higher levels of Kelcoloid passed 2 or 3 stools daily which were soft in texture but were fairly well formed. There did not seem to be any indications of a chronic toxicity. The eyes of the cats were clear, and the cats kept themselves neat and clean. Inspection of the organs after necropsy, both macro- and microscopic, of both control and

Kelcoloid fed cats indicated that the lesions which were found were of no significance insofar as any specific disease process was concerned. Kelcoloid appeared to be a wholesome food.

Discussion. The algins are partly digestible, but it is not known whether the animal directly or bacteria in the intestinal tract utilize these products. This is of no great nutritional significance since the products are used in only very small quantities in foods.

The difficulties encountered with diets containing high levels of algin may be attributed to the high water absorbing and bulking capacities of this high viscosity product. At the levels fed, water would produce a sticky, heavy paste or semi-gel causing difficulty in chewing and swallowing or a reduction or normal nutrient intake. This kind of physical effect may be expected with any product of this type. Nilson and Schaller(2) reported similar conditions when Irish moss was fed to rats.

In commercial usage the products are, of course, fully hydrated and used at levels of 0.1 to 0.5%. Thus the lowest level fed in these experiments, namely 5%, represents 10 to 50 times the amount that would be consumed in the daily diet of humans if everything eaten contained algin.

Conclusions. The data indicate that these algin products are free from any deleterious substances which adversely affect health when fed at levels which are at least 10 times higher than when used in foods or pharmaceutical products. The difficulties experienced with feeding the higher levels seem to be due, at least in greater part, to the physical texture imparted to the diets fed. This should not be a problem in the commercial use of the product. The data indicate that the three algin products which were tested are wholesome.

Received January 16, 1951. P.S.E.B.M., 1951, v76.

Reversible Agglutination of Trypsin Treated Erythrocytes by Normal Human Sera.* (18581)

Martin C. Rosenthal† and Lawrence I. Schwartz.
(Introduced by Mario Stefanini)

From the Ziskind Laboratories of the Joseph H. Pratt and New England Center Hospitals and the Department of Medicine, Tufts College Medical School, Boston.

Human erythrocytes, when treated in various ways, are susceptible to agglutination by apparently normal, compatible human sera. This effect is entirely independent of the usual blood groups and their agglutinins. Thomsen (1) first demonstrated that red cells exposed to certain bacteria can be agglutinated by most human sera. Friedenreich(2) extended these observations by showing that an enzyme derived from the bacteria was responsible for the change in the properties of the red cell. Since then it has been shown that exposure of red cells to Cholera Vibrio filtrates(3), viruses of the mumps-influenza group(3), and periodate ions(4) renders these cells panagglutinable by human sera. That the agglutinins in human sera responsible for these effects are varied and distinct has been shown by Stewart(5) and Moskowitz and Treffers (6). Brief mention is made by Wheeler *et al.*

---

* This work was supported by grants in aid from the American Cancer Society, Mass. Division and the C. H. Hood Dairy Foundation.

† This work was performed during the tenure of a Damon Runyon Clinical Research Fellowship, administered by the American Cancer Society.

1. Thomsen, O. Z., *Immun. Forsch.*, 1927, v52, 85.

2. Friedenreich, V., *Compte Rendu. Soc. Biol.*, Paris, 1928, v98, 894.

3. Burnet, F. M., and Anderson, S. G., *Aust. J. Exp. Biol. and M. Sci.*, 1947, v25, 213.

4. Burnet, F. M., In Briody, B. A., *J. Immunol.*, 1948, v59, 115.

(7) of the fact that erythrocytes exposed to the action of trypsin may be agglutinated by normal sera. They recognized that such an effect may not be entirely non-specific but may be related to the agglutination described by Thomsen. During the course of investigating the properties of trypsin-modified red cells, we have observed that these cells are capable of agglutination upon exposure to most human sera. No question of the usual compatibilities existed since this effect was noted even when the trypsinized cells were exposed to their own serum.‡ Agglutination titers of this sort ranged from 1 to 32 units. The character of the agglutinates was distinctive. They formed solid aggregates in undiluted or weakly diluted sera, and finer clumps with higher dilutions. They appeared only if the serum-cell mixtures were spun soon after contact. Incubation of serum-cell mixtures at room temperature for one hour did not produce spontaneous agglutination, nor did centrifugation then reveal agglutination. Observation of the agglutinates formed after immediate centrifugation revealed that they broke up spontaneously and became redispersed on continued contact with the serum mixtures. It appeared then that the agglutination observed was evanescent. The properties of the serum agglutinin as well as of the agglutination reversal effect were then studied.

Methods and materials. Blood samples were drawn from the antecubital vein and allowed to clot at room temperature. The serum was separated from the clot which was used to make the cell suspensions. Both cells and serum were used within 3 hours. In order to insure perfect compatibility, cells from an individual were used against his own serum.

Technic of trypsin modification of the erythrocyte. A modification of the method of Wheeler *et al.*(7) was used. Instead of whole blood, twice washed erythrocytes were used to make up 2% cell suspensions.§ Following exposure to 0.1% crude trypsin (Difco 1:250, product No. 8152) for 10 minutes at 37°C, the erythrocytes were twice washed again with .85% sodium chloride and resuspended to their original volume.

Agglutination tests. Agglutination tests were performed by adding two drops of the trypsin modified cell suspension to two drops of serum or saline dilutions of serum. Following one to 2 minutes incubation at room temperature, the tubes were centrifuged at 1500 R.P.M. for one minute and read for agglutination. The tubes were then vigorously tapped to redisperse the agglutinates as completely as possible. The mixtures were then incubated at room temperature for 5 minutes, recentrifuged, reread, and redispersed. This was repeated at 5 minute intervals for the first 30 minutes, and at 10 minute intervals for the last 30 minutes. As controls, diluted serum and 2% trypsin modified erythrocytes were held apart at room temperature and mixed at the end of an hour (Control A). In Control B, trypsin modified erythrocytes in contact with the serum-saline mixtures but undisturbed were also held for a similar period. Both sets of controls were then centrifuged and read for agglutination. In order to ascertain whether loss of agglutination was due to loss of agglutinin or to an alteration in the trypsin modified erythrocytes, these cells were kept in contact with serum dilutions until agglutination could no longer be demonstrated. The tubes were then centrifuged for 5 minutes and the serum dilutions carefully pipetted off into another set of tubes. To each residual button of red blood cells, 2 drops of fresh undiluted serum were added. To the removed serum dilutions, 2 drops of fresh trypsin modified cells were added. Both sets were shaken, incubated at room temperature for one minute and then centrifuged and read for agglutination. All agglutinations were read with the aid of a hand lens by one observer to insure uniformity.

---

5. Stewart, F. S., *J. Path. Bact.*, 1949, v61, 456.

6. Moskowitz, M. and Treffers, H. P., *Science*, 1950, v111, 717.

7. Wheeler, W. E., Luhby, A. L., and Scholl, M. L. L., *J. Immunol.*, 1950, v65, 39.

‡ No patient was included in this study in whom complete or incomplete iso- or auto-antibodies could be demonstrated by the usual technics.

§ The use of washed cells rather than whole blood resulted in a greater trypsin action, since the antitryptic activity of plasma is not encountered.

TABLE I. Trypsin Modified Erythrocytes and Serum Dilutions Respun at Stated Intervals.

Time after initial contact, min.		Serum dilutions					
	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
1	+++	++	++	±	—	—	—
5	+++	++	++	±	—	—	—
10	+++	++	++	±	—	—	—
15	—	++	++	±	—	—	—
20	—	±	++	±	—	—	—
25	—	±	+	±	—	—	—
30	—	—	+	±	—	—	—
40	—	—	±	±	—	—	—
50	—	—	—	±	—	—	—
60	—	—	—	—	—	—	—
Control A	+++	++	++	+	—	—	—
Control B	—	—	—	—	—	—	—

TABLE II. Determination of Serum Activity and Reactivity of Trypsin Modified Erythrocytes After 1 Hour Incubation.

		Serum dilutions					
	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
Residual button of serum-exposed* trypsin modified erythrocytes + 2 drops fresh undiluted serum	—	—	—	—	+	+++	+++
Removed serum-saline mixtures + 2 drops fresh trypsin modified erythrocytes	+++	+++	+	+	—	—	—

* Serum dilutions to which erythrocytes were exposed 1 hr are indicated by the heading.

Results. The results of the first procedure (Table I) show that with the passage of time, agglutination disappears first in the tubes with the highest serum content and then eventually from all the tubes. Admixture of the trypsin modified cells and serum dilutions *held apart* for a similar period (Control A) shows no such loss of agglutination. However, trypsin modified cells and serum dilutions *held together* but undisturbed (Control B) show a loss of agglutinability. It was evident that contact between cells and serum was necessary for loss of agglutination. Centrifugation was not an important factor.

The results of the experiment designed to demonstrate whether loss of agglutination was due to loss of the serum agglutinin or to an alteration in the trypsin modified erythrocytes are expressed in Table II. It may be seen that the agglutinin had not been lost in the serum dilutions. Rather the trypsin modified erythrocytes exposed to the first four serum dilutions and to a lesser extent to the fifth serum dilution had undergone an alteration making them unresponsive to the further action of the serum agglutinin. A critical amount of serum is necessary for this effect to occur since cells exposed to serum dilutions above 1:32¶ did not show this alteration at the end of one hour. It cannot be stated whether the serum contains a factor that directly brings about this altered reactivity of the treated erythrocytes or whether it accelerates a change on the red cell surface which occurs spontaneously at a slow rate.

Further observations on this agglutination reversal effect revealed that elevation of the temperature under which the effect was studied hastened the redispersal of the agglutinates while lowered temperatures inhibited this action. A study on the effect of various physical and chemical agents on the agglutinin and the agglutination reversal effect demonstrated certain differences between the two. The agglutinin was non-dialysable, destroyed by heating to 65°C for one hour, and only weakly active at extremes of pH. In contrast, the agglutination reversal effect was lost through dialysis, was thermostabile, and was still active in markedly alkaline serum. The agglutinin could be absorbed from serum with trypsin modified cells and then eluted into saline. It was not possible to do this with respect to the agglutination reversal effect.

¶ That dilution of normal sera to 1:32 often resulted in a loss of agglutination activity as well as loss of the agglutination reversal effect was coincidental. Sera from certain ill individuals have demonstrated an agglutination titer against trypsin modified cells as high as 1:256 although the agglutination reversal effect was seen only up to serum dilutions of 1:32.

Studies on the trypsin modified cells before and after exposure to serum demonstrated that they lost only their reactivity to the panagglutinin found in most sera. Their ability to react with other hemagglutinins, either complete, or incomplete, was unimpaired. Thus they are perfectly adequate as test cells for the detection of hyperimmune antibodies(8).

Discussion. The presence of agglutinins in most human sera, active against modified erythrocytes, may at times be of pathologic significance. Burnet and Anderson have suggested that *in vivo* modification of red cells by exposure to viruses or bacteria may induce a hemolytic episode. Similarly it is possible that proteolytic enzymes released from necrotic, neoplastic, or infected tissue may modify the contiguous erythrocytes in such a fashion as to render them susceptible to the action of agglutinins present normally in all sera. The modification of these cells in itself may in turn stimulate the formation of more agglutinins although it is not certain that such agglutinating bodies develop initially in response to an antigenic stimulus such as virus altered erythrocytes(9).

Practically, the ability of most normal sera to agglutinate trypsin modified red cells offers no deterrent to their use in searching for abnormal antibodies of the so-called blocking or incomplete type. We have successfully used these cells in the investigation of sera from erythroblastotic mothers and cases of acquired hemolytic anemia. One must, however, observe the precaution of incubating the cell-serum mixtures for at least an hour and preferably at 37°C in order to obviate the effect described above.

Summary. An agglutinin is described which exists in most normal sera and which is capable of agglutinating trypsin modified erythrocytes. By virtue of such action, it resembles the agglutinins active against erythrocytes altered by bacterial filtrates, viruses, and periodate ions. Undiluted serum or serum in weak dilution is able to reverse the activity of this agglutinin. Such a reversal effect is mediated through an altered reactivity of the trypsin treated erythrocytes with respect to the agglutinin described above. No modification of the reactivity of the cells with respect to other agglutinins has been noted. The effect of various physical and chemical agents on the activity of the agglutinin and the agglutination reversal effect is described and the patho-physiological role of the agglutinin is discussed.

---

8. Morton, J. A., and Pickles, M. M., *Nature,* 1947, v159, 779.

9. Lind, P., and McArthur, N., *Aust. J. Biol.,* 1947, v25, 247.

Received January 18, 1951. P.S.E.B.M., 1951, v76.

Effect of Prolactin on Phosphorus Metabolism of Pigeon Crop-Sac: P^{31} and P^{32} Analyses.* (18582)

ROBERT W. BROWN, DIXON M. WOODBURY, AND GEORGE SAYERS.

From the Departments of Pharmacology and Obstetrics and Gynecology, University of Utah College of Medicine, Salt Lake City.

Prolactin induces a proliferative hypertrophy of the pigeon crop-sac(1,2). Since tissue growth is associated with an increased synthesis of nucleic acids(3) and probably of other phosphorus-containing compounds, experiments were undertaken to determine the effect of prolactin on the phosphorus metabolism of the crop-sac. Special attention has been given to the quantitative relationship between the dose of prolactin and the phosphorus concentration of the crop-sac because of our interest in the development of a new method for the bioassay of the hormone.

Methods. Prolactin† was administered intramuscularly in equal doses once daily for 4 days to groups of 5 to 15 White Carneau pigeons, 6 to 8 weeks of age. Twenty-four hours after the last injection of prolactin, a solution of inorganic P^{32} which was obtained from Oak Ridge was diluted with 0.9% sodium chloride (100 microcuries per ml) and administered intraperitoneally in a dose of 100 microcuries per kg of body weight. At an appropriate time after the injection of P^{32} the birds were sacrificed by decapitation, blood was collected and two portions of the crop-sac, each weighing 200 to 300 mg, were excised for analysis of P^{31} and P^{32}. One portion of tissue was immediately frozen in solid carbon dioxide for the analysis of various phosphate fractions. The other portion was ashed in 10 N sulfuric acid, the oxidation being completed by the dropwise addition of concentrated nitric acid. Total P^{31} concentration was determined by the Fiske-Subbarow method(4). An aliquot of the ashed material was evaporated in a cupped planchet and the total P^{32} activity measured in a Geiger-Müller counter. The frozen tissue was homogenized in cold 5% trichloracetic acid. Total P^{31} and P^{32} concentrations‡ of the acid-soluble material were determined by the procedures just described. Inorganic P^{31} of the acid-soluble fraction was determined by direct application of the Fiske-Subbarow method(4) to an aliquot of the supernatant fluid. Inorganic P^{32} was measured by the calcium hydroxide method described by Dziewiatkowski and Bodian(5). In the analyses of inorganic P^{31} and P^{32} a small amount of adenosinetriphosphate and phosphocreatine may have been hydrolyzed; the phosphorus so liberated would then be included in the inorganic acid-soluble fraction. Precautions were taken to reduce such hydrolysis to a minimum(6). P^{31} and P^{32} concentrations of the organic acid-soluble fraction were calculated as the difference between total and inorganic acid-soluble concentrations. Phospholipids were extracted from the acid-insoluble residue with an alcohol-ether mixture. The P^{31} concentration of this fraction was determined on an ashed aliquot of the extract by the Fiske-Subbarow procedure(4). An-

---

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service. The P^{32} was received on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

1. Riddle, O., and Braucher, P. F., *Am. J. Physiol.*, 1931, v97, 617.

2. Riddle, O., Bates, R. W., and Dykshorn, S. W., *Am. J. Physiol.*, 1933, v105, 191.

3. Caspersson, T., *Symp., Soc. Exp. Biol.*, 1947, no. 1, 127.

† Prolactin (potency, 20 to 25 I.U. per mg) was generously supplied by Dr. R. W. Bates, E. R. Squibb and Co.

4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

‡ P^{32} concentration as used in this paper is the activity in counts per minute per gram of tissue.

5. Dziewiatkowski, D., and Bodian, D., *J. Cell. and Comp. Physiol.*, 1950, v35, 141.

6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism, 2nd Edition, Burgess Publishing Co., Minneapolis, Minn., 1949.

other aliquot was evaporated in a cupped planchet and radioactivity measured. P^{31} and P^{32} concentrations in the residue fraction were determined by methods described above for total phosphorus. Plasma inorganic P^{31} and P^{32} were determined by the methods described above for the crop-sac.

All radioactivity determinations were compared with a standard P^{32} sample prepared from an aliquot of the material which was injected. The standard was counted simultaneously with the tissue and plasma samples and corrections for P^{32} decay were applied. Specific activity is expressed as P^{32} counts per minute per g tissue divided by mM P^{31} per kg tissue.

Results and discussion. The temporal aspects of P^{32} uptake by the crop-sac were studied in two groups of pigeons. One group received no prolactin and another group received a total of 10 mg of the hormone, a dose capable of producing maximum stimulation of the crop-gland. Both groups were injected with P^{32} and sacrificed 1, 4 and 8 hours after administration of the isotope. The results are shown in Fig. 1. In each group, uptake of P^{32} remained constant over the period studied. The data show that both the rate and the magnitude of uptake of P^{32} increased threefold under the influence of prolactin. From the standpoint of convenience, the pigeons were sacrificed 8 hours after the administration of P^{32} and this interval was employed in all subsequent experiments.

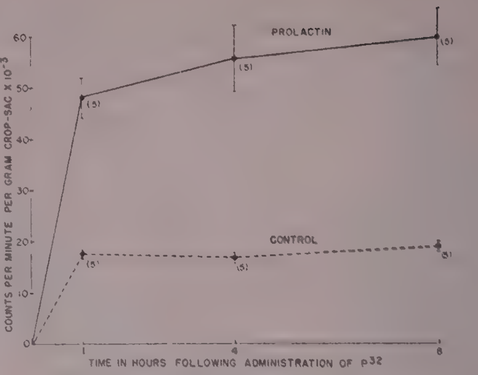


FIG. 1.

P^{32} uptake in control pigeons and pigeons injected with a total dose of 10 mg prolactin. The thin vertical bars represent standard errors. The figures in parentheses represent the number of birds.

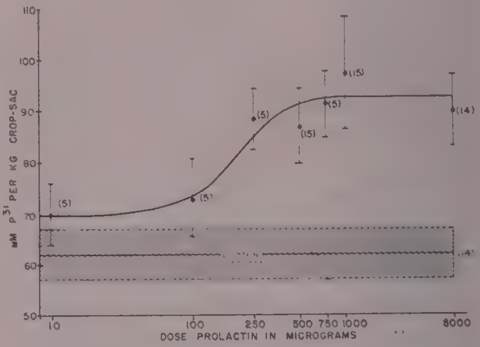


FIG. 2.

Effect of various doses of prolactin on concentration of total P^{31} in pigeon crop-sac. The shaded area above and below the control value and the thin vertical lines about the average values for the prolactin-treated pigeons represent one standard deviation. The doses are plotted on a log scale. The figures in parentheses represent the number of birds.

The effect of prolactin on the total P^{31} content of the crop-sac is graphically illustrated in Fig. 2. The average concentration of P^{31} in the crop-sacs of untreated pigeons is 62 mM P^{31} per kg tissue. A total dose of 10 μg of prolactin produced a small but significant increase in the concentration of total phosphorus in the crop-sac ($p = 0.02$). This is a much smaller dose of prolactin than can be detected by other assay technics with the exception of the intracutaneous assay method described by Lyons and Page(7). The specificity of the intracutaneous assay procedure has been challenged(8). A total dose of 100 μg did not induce a significantly greater increase in the concentration of P^{31} in the crop-sac than did 10 μg. There was a sharp rise in the log-dose response curve between the 100 and 250 μg total dose levels. Maximum response was attained between the 250 and 1000 μg total dose levels; further study is required to determine accurately the maximally effective dose. The 10 and 100 μg total doses produced no macroscopic evidence of

---

7. Lyons, W. R., and Page, E., PROC. SOC. EXP. BIOL. AND MED., 1935, v32, 1049.

8. Lahr, E. L., Bates, R. W., and Riddle, O., *Endocrinology*, 1943, v32, 251.

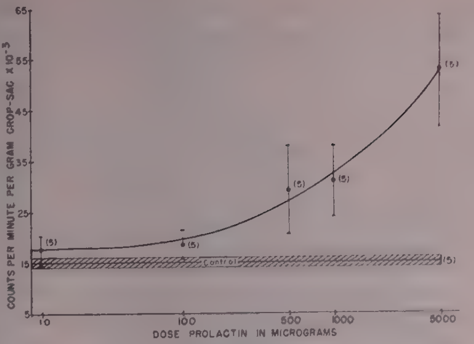


FIG. 3.

Effect of various doses of prolactin on the uptake of P^{32} by pigeon crop-sac. The shaded area above and below the control value and the thin vertical lines about the average values for the prolactin-treated pigeons represent one standard deviation. The doses are plotted on a log scale. The figures in parentheses represent the number of birds.

enhanced vascularization or cellular proliferation, although the total phosphorus concentration of the crop-sac increased. It is probable that metabolic processes are accelerated before there is a detectable increase in the weight of the organ.

The effect of various doses of prolactin on the uptake of P^{32} by the crop-sac is depicted in Fig. 3. A total dose of 10 μg produced no significant increase and 100 μg increased only slightly the uptake of the isotope. However, total doses greater than 100 μg produced marked increases in P^{32} uptake; 5000 μg more than tripled the uptake of P^{32} by the crop-sac. Larger doses must be injected in order to determine the quantity of prolactin which induces a maximum response.

The data suggest that the effect of prolactin on the P^{31} and P^{32} concentrations of the crop-sac is a potentially useful phenomenon for the bioassay of the hormone. Preliminary studies indicate that the results are reproducible. At present, it is not possible to state whether P^{31} or P^{32} determination offers the most useful method of assay. Also the specificity of the methods must be established by the determination of the effect of other adenohypophyseal hormones on the phosphorus metabolism of the crop-sac. The results of the analyses of P^{31} and P^{32} in various phosphate fractions are shown in Fig. 4. Pigeons which received 100 microcuries of P^{32} per kg body weight are compared with birds injected with the same dose of P^{32} plus a total dose of 500 μg prolactin. This dose of prolactin produces gross proliferation of the crop-sac. The following tentative interpretations of the results are offered merely as working hypotheses and are presented chiefly for the purpose of stimulating additional research on the subject.

Prolactin increases the rate of turnover of P^{32} without influencing the inorganic P^{31} concentration of the acid-soluble fraction. Calculations based on the concentrations of plasma inorganic P^{31} and P^{32} (assuming an extracellular space equivalent to 20% of body weight) indicate that the concentration of inorganic P^{31} in cell water is approximately 10 times the extracellular inorganic P^{31} concentration and that the specific activity of the intracellular inorganic fraction is approximately the same as the specific activity of the tissue. (Tissue specific activity represents the sum of the specific activity of the extracellular and intracellular compartments.) This suggests that the overall increase in specific activity of phosphate induced by prolactin can be accounted for almost entirely by changes in the rate of turnover of this element in the intracellular compartment. No change took place in the specific activity of the plasma of prolactin-treated pigeons. However, since a maximally stimulated crop-gland

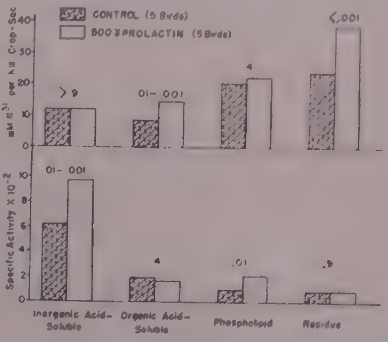


FIG. 4.

Effect of a total dose of 500 μg prolactin on the concentration of P^{31} and the specific activity of phosphorus in various phosphate fractions of the pigeon crop-sac. The p value immediately above each column expresses the statistical significance of the change induced by prolactin.

represents only 1% of the total body weight of a pigeon, the changes in phosphorus turnover in the crop-sac may not be expected to influence appreciably the large reservoir of phosphate present in the extracellular fluid. The data suggest that inorganic phosphorus enters the cells of the stimulated gland at an increased rate and serves as the source of phosphate for the synthesis of organic phosphorus compounds. This is in conformity with the data to be presented below.

Prolactin produces an increase in the concentration of organic P^{31} in the acid-soluble fraction without influencing the specific activity. This suggests that prolactin increases the rate of synthesis of one or more of the phosphorylated intermediates included in this fraction. Prolactin increases the specific activity in the phospholipid fraction without changing its concentration of P^{31}. The role of phospholipid in the metabolism of fat is uncertain. The data could be interpreted to mean that phospholipid is involved (a) in the transport of fat to the crop-sac [see Chaikoff(9) for convincing evidence against such a role], or (b) in the intracellular synthesis and or oxidation of fat. It is of interest in this connection that both the cells(10) and the milk(11) of the crop-sac contain high concentrations of lipid material.

Prolactin increases the concentration of P^{31} in the residue fraction of the crop-sac without causing an associated change in specific activity. This suggests an increase in the synthesis of nucleoproteins; such an increase is expected in a gland which has undergone proliferation and is in agreement with the demonstration of McShan *et al.*(12) that prolactin increases the concentration of nucleic acids in the crop-sac.

Summary. Prolactin increases the concentration of total P^{31} and P^{32} in the pigeon crop-sac. The applicability of these changes to the bioassay of the hormone is discussed.

Prolactin (a) increases the specific activity without affecting the concentration of inorganic acid-soluble phosphorus in the crop-sac, (b) increases the concentration without affecting the specific activity of the organic acid-soluble fraction, (c) increases the specific activity without affecting the phosphorus concentration in the phospholipid fraction, and (d) increases the concentration without affecting the specific activity of phosphorus in the residue fraction. The significance of these results is discussed.

The authors take this opportunity to thank Dr. Louis S. Goodman for the many suggestions and criticisms which he presented during the course of this study.

---

9. Chaikoff, I. L., and Entenman, C., *Ann. Rev. Biochem.*, 1948, v17, 253.

10. Beams, H. W., and Meyer, R. K., *Physiol. Zoöl.*, 1931, v4, 480.

11. Davies, W. L., *Biochem. J.*, 1939, v33, 898.

12. McShan, W. H., Davis, J. S., Soukup, S. W., and Meyer, R. K., *Endocrinology*, 1950, v47, 274.

Received January 24, 1951. P.S.E.B.M., 1951, v76.

Uric Acid Diabetes in the Rat.* (18583)

R. R. GRUNERT† AND P. H. PHILLIPS.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin.

An investigation into the susceptibility of the albino rat to uric acid diabetes was prompted by the recent reports of Griffith (1,2) that methionine deficient rabbits exhibited diabetic symptoms following the injection of uric acid. When rabbits, which re-

---

* Published with the approval of the Director of the Wisconsin Agri. Exp. Sta. Supported in part by a grant from the Salt Producers Association.

† Formerly Postdoctorate Fellow, National Institutes of Health. Present address, Biological Laboratory, DuPont Co., New Brunswick, N. J.

1. Griffiths, N., *J. Biol. Chem.*, 1948, v172, 853.

2. Griffiths, N., *J. Biol. Chem.*, 1950, v184, 289.

ceived a complete ration (stock ration or the methionine deficient ration supplemented with that amino acid), were used no hyperglycemia resulted. Uric acid had been reported non-diabetogenic(3-5) but normal animals were used. The problem, therefore, resolved itself into testing the diabetogenic activity of uric acid in methionine deficient rats.

Methods and materials. Methionine deficient rats were obtained by feeding a ration composed of 18% protein (α-protein and oxidized casein),[‡] 73% sucrose, 4% Salts IV (6), 5% corn oil and all the known vitamins. A similar ration which contained only 9% casein (81% sucrose) was also used. The control rations resulted from the supplementation with 0.3% dl-methionine (α-protein and 9% casein) and 0.6% dl-methionine (oxidized casein) and the use of 18% casein as the source of protein. When a sodium deficient ration was desired, the above salts were replaced by 2.5% sodium-potassium low Salts IV(7), the sucrose increased to 74.5% and the ration supplemented with 0.25% potassium as the bicarbonate. The raw soyabean ration was composed of 59% corn, 34% raw soyabean flakes, 5% alfalfa and 2% Salts IV. Male weanling rats (Holtzman) were placed on the experimental rations described above, for the lengths of time indicated in Tables I to IV. Then they were either subjected to injections of uric acid (Tables I and III) or their tissues were analyzed for glutathione (Tables II and IV). Those subjected to the uric acid treatment received an intraperitoneal injection of an aqueous suspension (75 to 350 mg/cc depending on the dosage). The blood sugar values were determined at 0, 2, 4 and 6 days after the initial injection of uric acid. The method of Folin and Malmros as adapted for the Evelyn colorimeter by Horvath and Knehr(8) was used. Successive uric acid injections were given as indicated in Tables I and III. The α-protein rats were fasted 12 hours before each bleeding and 15 hours before the uric acid injections. The sodium deficient rats were not fasted before the bleedings or uric acid injections. Blood, liver and kidney glutathione values were determined in all animals not subjected to uric acid injection according to the nitroprusside method as modified by Grunert and Phillips(9).

TABLE I. Effect of Intraperitoneal Injections of Uric Acid on Blood Sugar of Rats Fed a Methionine Deficient Ration (α-protein).

		Blood sugar[*]	
Time interval, days	Uric acid dosage, mg/kg	α-protein, mg %	α-protein + methionine, mg %
0	200	90 ± 10[†]	88 ± 7
2	—	92 ± 5	99 ± 4
4	500	106 ± 9	93 ± 6
6	—	97 ± 5	107 ± 8

[*] Fasting levels (12 hr).

[†] Stand. error of the mean, 4 rats.

Results. The report that α-protein was low in methionine(10) led to its use for producing methionine deficient rats. Two groups of rats (4 animals each) received as their protein supplement 18% α-protein and 18% α-protein plus 0.3% dl-methionine for 15 weeks. They were injected with uric acid at that time following a 15-hour fast. The blood sugar was determined before and at two day intervals after the injection. The results are shown in Table I. Two days after the intraperitoneal injection of 200 mg/kg of uric acid the blood sugar was unchanged. The use by Griffith of 1 g/kg in the rabbit(1,2) and the ratio of dosages of alloxan used in the rat

---

3. Dunn, J. S., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. and Bact.*, 1943, v55, 245.

4. Gitter, S., and Cardeza, A. F., *Rev. Soc. argent de biol.*, 1946, v22, 353.

5. Thorogood, E., *Fed. Proc.*, 1944, v3, 48.

[‡] The α-protein was a purified protein fraction from soyabeans (Glidden Co., Chicago) which supplied 0.3% methionine when fed at 18% of the ration. In comparison, 18% casein supplies 0.6% methionine. The oxidized casein(13) ration was supplemented with 0.25% dl-tryptophane.

6. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, v109, 657.

7. Grunert, R. R., and Phillips, P. H., *J. Biol. Chem.*, 1949, v181, 821.

8. Horvath, S. M., and Knehr, C. A., *J. Biol. Chem.*, 1941, v140, 869.

9. Grunert, R. R., and Phillips, P. H., *Arch. Biochem.*, in press.

10. Grau, C. R., and Almquist, H. J., *J. Nutrition*, 1943, v26, 631.

TABLE II. Effect of a Methionine Deficiency, Induced by 9% Casein, α-protein and Oxidized Casein on the Blood and Liver Glutathione Levels of the Rat.

Ration	Time of exp., wk	No. of animals		Blood glutathione		Liver glutathione	
			.3% dl-methionine	mg %	.3% dl-methionine, mg %	mg/100 g	.3% dl-methionine, mg/100 g
9% casein	4‡	8	9	27.1 ± 2.5*	23.5 ± 1.7	43 ± 3	179 ± 7
18% α-protein	15	4	4	39.6 ± 2.6	34.3 ± 0.8	47 ± 9	126(2)
18% oxidized casein	2§	8	8	41.2 ± 2.8	41.6† ± 2.3	60 ± 4	109† ± 12
Raw soyabean meal	6	6	—	21.1 ± 0.7	—	138 ± 9	—
18% casein	6	27, 15	—	32.5 ± 0.8	—	111 ± 6	—

* Stand. error of the mean.
† Supplemented with .6% dl-methionine.
‡ Four weeks, following a 2-wk interval on 18% casein.
§ Two weeks, following a 4-wk interval on 18% casein.

TABLE III. Effect of Intraperitoneal Injections of Uric Acid on Blood Sugar Levels of Rats Fed a Sodium Deficient Ration.

Time interval, days	Uric acid dosage, mg/kg	Blood sugar*	
		Sodium deficient mg %	Control, mg %
0	250	—	—
2	500	139 ± 16†	128 ± 3
4	1000	121 ± 8	108 ± 1
6	—	123 ± 2	131 ± 5
0	1000	—	—
2	—	105 ± 7	115 ± 6

* Non-fasting levels.
† Stand. error of the mean, 4 rats.

and rabbit(11) led to the use of this level of uric acid. On the fourth day, with the blood sugar still normal a second injection of uric acid (500 mg/kg) was given without effect. There was apparent pain in the rats following the injection of uric acid, and this disappeared in a few hours.

The fact that uric acid appeared nondiabetogenic in the methionine deficient rat suggested that the tissue levels of glutathione may not have been lowered sufficiently. Accordingly the effect of the α-protein and other methionine deficient rations on blood and liver glutathione was studied. The results are shown in Table II. The liver glutathione of the rats which received the methionine deficient rations (9% casein, 18% α-protein and 18% oxidized casein) was decreased to less than 50% of the normal value of 111 mg/100 g. Supplementation of the deficient rations with methionine permitted the maintenance of normal levels of liver glutathione in the α-protein and oxidized casein rats and an increased level (179 mg/100 g) in the 9% casein rats. The blood glutathione reacted differently. It tended to vary with the type of ration used, as indicated in Table II. When α-protein and oxidized casein were fed the blood glutathione increased to 39.6 and 41.2 mg % respectively. It seems that low methionine rations cause elevated dl-methionine concentrations in the blood. The blood glutathione of rats maintained on α-protein for 7 months was 42.0 mg % compared to 32.1 mg % for the methionine supplemented rats, while the liver glutathione was 65.2 mg/100 g compared to 144 mg/100 g respectively.

The effect of uric acid on the blood sugar of sodium deficient rats was studied because of their low blood glutathione values(7,12) and the insistence of previous workers(2,13) for this criterion in developing uric acid diabetes. The results are shown in Table III.

The need for decreased levels of glutathione in both the liver and blood in order to demonstrate the diabetogenic activity of uric acid became apparent from the resistance of sodium deficient (low blood glutathione) and methionine deficient (low liver glutathione) rats to uric acid diabetes. An attempt was therefore made to lower the level of blood and liver glutathione simultaneously by super-

---

11. Lukens, F. D. W., *Physiol. Rev.*, 1948, v28, 306.

12. Grunert, R. R., Meyer, J. H., and Phillips, P. H., Forthcoming publication.

13. Bennett, M. A., and Toennies, G., *J. Biol. Chem.*, 1942, v145, 671.

TABLE IV. Blood, Liver, and Kidney Glutathione Levels as Affected by the Nutritional State of the Rat.

Nutritional state of rat	Tissue glutathione		
	Blood, mg %	Liver, mg/100 g	Kidney, mg/100 g
Control	32.5 ± 0.8*	111 ± 6	99 ± 6
Sodium deficient	15.3 ± 1.6	150 ± 9	98 ± 7
Methionine deficient (9% casein)	27.1 ± 2.5	43 ± 3	88 ± 2
” ” (18% oxidized casein)	41.2 ± 2.8	60 ± 4	80 ± 4
Na + methionine (9% casein) deficient	17.2 ± 0.8	141 ± 9	107 ± 2
Na + methionine (oxidized casein) deficient	31.8 ± 1.7	58 ± 7	73 ± 6

* Stand. error of the mean, 8 to 10 rats per group except controls where 27 rats used for blood glutathione and 15 for liver and kidney glutathione.

imposing a methionine deficiency on top of a sodium deficiency. The results are shown in Table IV. The levels of kidney glutathione are included. The data from Table II on the effect of the 9% and 18% oxidized casein on blood and liver glutathione are also included for more direct comparison. As reported previously(12) the blood glutathione of sodium deficient rats was decreased (15.3 mg %) while the liver glutathione was elevated to 150 mg/100 g. When the 9% casein ration was superimposed on the sodium deficiency for the last 4 weeks of the 6 weeks experimental period the blood glutathione remained low at 17.2 mg % and liver glutathione remained elevated at 141 mg/100 g. When the oxidized casein ration was superimposed on the sodium deficiency for the last two weeks of the 6 weeks experimental period the blood glutathione returned to normal (31.8 mg %) and the liver glutathione decreased to 58 mg/100 g. The complete absence of methionine in oxidized casein(13) apparently decreased the requirement for sodium by virtue of a lack of growth and thereby eliminated the effect of sodium.

Discussion. The resistance of methionine deficient and sodium deficient rats to uric acid diabetes was thought at first to be in contradiction to the work of Griffith(1,2). The necessity for a low level of liver glutathione thus permitting the conversion of uric acid to some toxic agent (alloxan?) and a simultaneously low blood glutathione level to permit the passage of the agent to the pancreas was, however, realized. On this basis the sodium deficient rats were non-susceptible because of the normal metabolism of uric acid in the liver (normal glutathione content). The methionine deficient rats were resistant because of the high blood glutathione content which would undoubtedly tie up the toxic agent (alloxan?) formed by the sulfhydryl deficient liver.

The failure to obtain a nutritional state in which both the blood and liver glutathione levels were simultaneously reduced does not permit conclusive statements as to diabetogenic activity of uric acid in the rat. However it should be noted that Williams and Bailey(14) failed to confirm Griffith's work with rabbits. Furthermore, uric acid has been found to be converted quantitatively to allantoin by the sulfhydryl deficient livers from rats maintained on α-protein and oxidized casein(15). Because of this report it is felt that when and if a simultaneous lowering of blood and liver glutathione in the rat is obtained uric acid will still be non-diabetogenic.

Summary. Uric acid was non-diabetogenic in methionine and sodium deficient rats. Methionine deficiencies induced by 9% casein, α-protein and oxidized casein rations lowered the liver glutathione but did not lower the blood glutathione. The superimposing of a methionine deficiency on a sodium deficiency (low blood glutathione) did not cause a simultaneous decrease in the blood and liver glutathione of the rat.

---

14. Williams, J., and Bailey, C. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 583.

15. Grunert, R. R., and Phillips, P. H., Forthcoming publication.

Received January 30, 1951. P.S.E.B.M., 1951, v76.

Effect of Irradiation and of Various Reagents on Viscosity of Nucleoprotein Solutions.* (18584)

ROBERT N. FEINSTEIN. (Introduced by J. M. Coon)

From the Toxicity Laboratory and the Department of Biochemistry, University of Chicago.

The effects of X-radiation in (a) producing chromosome breaks *in vivo* and (b) reducing the viscosity of nucleic acid and nucleoprotein solutions *in vitro* are well known. Because of the partially radiomimetic effects of H_2O_2, it was considered of interest to test the effects of H_2O_2 *in vitro* on nucleoprotein solutions, using the relative viscosity of the solutions as criterion of effect. Because of the known effects of cysteine and related compounds in reducing radiation mortality, a study was included of the effects of these compounds on nucleoprotein viscosity.

Experimental. Thymus chromosomes were prepared by the method of Mirsky and Ris (1), except that the final suspension in 0.14 M NaCl was made such that each ml represented one gram of initial thymus. Immediately before use, one volume of this suspension was mixed with an equal volume of N/10 NaOH. Of this 1:1 mixture, 5 ml were pipetted into a 25 ml Erlenmeyer flask containing 1 ml of the substance being tested. Molarities listed in the table are those of the agent as added; in the final mixture the molarity is thus 5/6 of that shown. Chemicals to be tested were used as received, with no attempt at purification. They were dissolved in slightly less than the proper volume of H_2O, brought to pH 8.0, and made to volume with H_2O. Materials of limited solubility were filtered before use.

Procedure. The mixture of 1:1 chromosomes-NaOH and agent to be tested was put into an Ostwald viscometer in a 37°C water bath. After an equilibration period of 15-20 minutes, the passage time was measured. The viscometers were kept in the 37° bath, and the passage time was again measured at the times shown.

Results. Effect of hydrogen peroxide. In preliminary experiments, the effect was tested of 0.1 M H_2O_2 on 5 volumes of thymus chromosomes solution. For comparison, 0.1 M NaCl was used. The viscosity of these solutions under the influence of H_2O_2 was consistently slightly higher than under the influence of the NaCl. In succeeding experiments, however, it was realized that if H_2O was used for comparison, then H_2O_2 was without effect, while 0.1 M NaCl or 0.1 M KCl reduced the viscosity slightly. These differences, however, were all so slight that this question was not further pursued.

Effect of Cysteine. The striking effect of cysteine on the viscosity of solutions of thymus chromosomes is indicated in the data of Tables I, II and III. There are included viscosity data taken at various times after mixture of the chromosome suspension with the NaOH and the cysteine solution, since this effect is considerably magnified with the passage of time.

Effect of the concentration of thymus on the "cysteine effect". As the alkaline solution of thymus chromosomes is further diluted with N/10 NaOH, the effect of cysteine in increasing the viscosity is lost. This is illustrated in Table I. This diminution in cysteine effect is apparently due to nucleoprotein (thymus chromosome) concentration rather than to the level of the initial viscosity, since preparations of varying initial viscosity lost the cysteine effect at the same degree of dilution, rather than at the same viscosity.

Effect of the concentration of cysteine on the "cysteine effect". Cysteine at 0.05 M or 0.06 M is ideal for demonstrating the effect of cysteine in increasing the viscosity of alkaline solutions of thymus chromosomes. If the cysteine concentration is decreased to 0.01 M or less, the viscosity increase is much less striking; if the cysteine concentration is increased to 0.1 M, the viscosity increase in a

---

* This work was carried out under a research contract with the Atomic Energy Commission.

1. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, v31, 1, 7.

TABLE I. Effect of Concentration of Thymus on the "Cysteine Effect."

Exp. No.	Incubation (hr)	Thymus dilution Agent added	Relative viscosity 1:1 CySH*	 1:1 H_2O	 1:125 CySH*	 1:125 H_2O	 1:2 CySH*	 1:2 H_2O
1	0		9.3	5.9	4.6	4.7	3.1	3.3
	1		14.5	6.3	5.1	4.8	3.1	3.3
	2		16.8	6.5	5.2	4.8	3.1	3.2
	4		19.2	6.5	5.3	4.8	3.1	3.2
2	0		11.1	7.2	5.9	5.7	3.6	3.6
	1		17.4	8.0	6.7	5.8	3.6	3.6
	2		21.4	8.2	6.9	5.8	3.7	3.7
	4		26.2	8.4	7.3	5.8	3.6	3.6

* CySH = cysteine.

TABLE II. Effect of Cysteine and Related Agents on Viscosity of Alkaline Solution of Thymus Chromosomes.

Incubation (hr)	Relative viscosity in presence of .05 M substance shown H_2O	Cysteine	Glutathione	Cystine*	Cyanide	Thiocyanate	Ascorbic acid	BAL	Thiourea	Urea	Thiouracil*	Uracil*
0	11.1	49.2	50.8	10.3	20.1				11.2			
4	14.4	116.0	>120	12.7	43.0				15.2			
0	5.3	6.6					5.1	5.3				
4	5.6	12.2					10.1	6.0				
0	12.5	15.7				14.0			12.3	12.0	12.4	11.7
4	13.9	33.4				25.9			14.6	14.6	14.0	14.7

* Cystine, uracil, and thiouracil were filtered saturated solutions, appreciably more dilute than the stated .05 M.

TABLE III. Effect of Cysteine on X-ray Reduction of Nucleoprotein Viscosity.

Sample No.	1	2	3	4
Treatment	X-rayed in presence of cysteine	X-rayed in absence of cysteine	Not X-rayed; cysteine added	Not X-rayed; cysteine not added
		(a) "Prophylactic" effect.		
Viscosity, zero time	10.4	6.8	11.3	11.5
3 hr	71.3	6.7	86.7	12.0
Sample No.	1	2	3	4
Treatment	X-rayed; cysteine added	X-rayed; cysteine not added	Not X-rayed; cysteine added	Not X-rayed; cysteine not added
		(b) "Therapeutic" effect.		
Viscosity, zero time	9.9	6.9	11.2	11.0
1½ hr	25.8	7.1	62.8	12.2

X-rayed in layer ca 1 cm deep in beaker covered with parafilm and packed in cracked ice. Administered 50000 r at 20 r per sec. 250 KV. 15 milliamperes. No filter used.

short time is so great as to make the use of Ostwald viscometers impractical.

Effect of time of incubation. It will be noted from Tables I and II that the viscosity of the cysteine-containing solutions increases with the passage of time. If the cysteine solution is replaced by water the rise in viscosity is extremely small, if present at all. Occasionally the viscosity in the presence of cysteine has been observed to rise continuously for several hours, and then, at about 4 hours, to begin to decrease. This phenomenon has been attributed to bacterial contamination, with subsequent degradation of the nucleoprotein.

It has also been observed that a given

saline (0.14 M NaCl) suspension of thymus chromosomes, kept in the ice box over a period of time, produced less and less viscous solutions when aliquots are removed and diluted with alkali. Although the decrease in viscosity thus produced is sometimes very large, it is not accompanied by any change in the protein concentration of the solution; that is to say, it does not represent bacterial or autolytic proteolysis. A possible explanation for the increased viscosity of alkaline solutions upon incubation lay in the effect of the repeated "shearing" occasioned by the repeated sucking up of the solutions through the viscometers; this might conceivably so orient asymmetric molecules as to increase viscosity. This was tested, however, and found to be not so; the mere process of determining the viscosity does not effect the viscosity.

Cysteine effect in molar salt solutions. All work described in the rest of this report was performed on 1:1 (v/v) mixtures of N/10 NaOH and chromosome suspensions. Mirsky and Ris(1) fractionated such chromosomes by placing them in a solution of 1 M NaCl. It was felt of interest to determine whether cysteine would increase the viscosity of such salt solution of chromosomes, as well as of alkaline solutions. This was found to be the case, but there was also observed some sedimentation in these preparations, hence alkaline solutions continued to be used. It is of interest that chromosomes diluted with 1 M NaCl were appreciably more viscous than if diluted with the same volume of 0.1 M NaOH.

Effect of other chemical agents on the viscosity of alkaline solution of thymus chromosomes. The other chemicals tried were those that have been tested as therapeutic or prophylactic agents for radiation toxicity and whose effects would seem in some way related to the reducing properties of cysteine. These data are summarized in Table II, in which, for the sake of brevity, only the initial and the 4-hour figures are given. For comparison, the effects of cysteine and water controls have been included.

Effect of cysteine on x-ray reduction of viscosity of chromosome solutions. For reasons discussed below, it was considered of interest to determine whether cysteine had any effect on the reduction of nucleoprotein viscosity by irradiation. In one experiment (Table IIIa), the cysteine was added to a portion of the alkaline nucleoprotein solution before X-radiation of the solution with 50,000 r. The data indicate that, percentage wise, the cysteine largely protects the viscosity of the solution. Using as the basis of calculations the "inherent viscosity" of the solute, *i.e.*, the relative viscosity of the solution minus one, it will be noted that 3 hours after completion of the irradiation, the viscosity of the solution irradiated in the absence of cysteine is 48% lower than its non-irradiated control, while the decrease in the presence of cysteine is 18%. In the next experiment (Table IIIb) the cysteine was added shortly after X-radiation with 50,000 r. In this case, after 1½ hours, the viscosity of the irradiated solution was 45% lower than its non-irradiated control in the absence of cysteine, and 60% lower in the presence of cysteine. In a third experiment, an attempt was made to determine whether the protective effect of cysteine diminished if the cysteine were added after a longer post-irradiation period. This proved not to be the case; rather the cysteine effect was greater if the cysteine were added later. This increased effect, however, is difficult to interpret, since the same is true in the case of non-irradiated nucleoprotein solution.

Discussion. The possibility was considered here that the effect of cysteine in increasing the viscosity of nucleoprotein solutions may be involved in the mechanism of cysteine protection against X-radiation lethality. Thus if X-ray sensitive nucleoproteins are in some way stabilized, *e.g.*, if the nucleic acids are somehow more highly polymerized, these nucleoproteins may be more resistant to irradiation. This suggestion, however, is not entirely borne out by the results reported. Thus thiourea, reported by Limperos and Mosher(2) to reduce X-ray mortality, does not increase the viscosity of alkaline solutions

---

2. Limperos, G., and Mosher, W. A., *Science,* 1950, v86, 112.

of thymus chromosomes, while thiocyanate, reported by Herve and Bacq(3) actually to *increase* X-ray lethality, was found here to increase nucleoprotein viscosity. On the other hand, most of the agents tested did have parallel activity in their effects on nucleoprotein viscosity and *in vivo* X-ray toxicity. Among these are cysteine(4,5), cyanide(6), and glutathione(5), which respond positively in both tests, and cystine(5), thiouracil (7,8), and ascorbic acid(5), which give negative results in both tests, although ascorbic acid does show a positive effect in the nucleoprotein viscosity test after a prolonged period of time.

In addition, the parallelism holds true for the experiments on the effect of cysteine on X-ray reduction of nucleoprotein viscosity. Patt *et al.*(4) have shown that cysteine protects rats from the lethal action of X-rays only if administered shortly before irradiation. Similarly, it was found here that cysteine protects nucleoprotein solution from viscosity reduction by X-rays much more effectively if added prior to the irradiation.

The possibility must remain, therefore, that some such phenomenon as this cysteine augmentation of nucleoprotein viscosity is a partial explanation of the reduction in X-ray lethality by cysteine and some other agents. The exceptions noted may represent entirely separate phenomena. Thiourea, for example, may protect against X-rays by reducing the metabolic level of the whole organism, in line with the ideas of Blount and Smith(7) and others. No explanation is here offered for the paradoxical behavior of thiocyanate.

Summary. 1. Cysteine, glutathione, cyanide, and thiocyanate greatly increase the viscosity of alkaline solutions of nucleoprotein (thymus chromosomes), while urea, uracil, thiourea, thiouracil, cystine, and BAL do not do so. Ascorbic acid increases the viscosity only after prolonged incubation. Hydrogen peroxide is without effect on the viscosity. 2. If cysteine is added to the alkaline nucleoprotein solution before X-radiation, the expected reduction in viscosity is largely prevented. If the cysteine is added after X-radiation, it is much less effective in this respect. 3. The suggestion is made that these phenomena may be relevant to the question of the mechanism of cysteine and some other agents in increasing resistance of organisms to X-ray lethality.

---

3. Herve, A., and Bacq, Z. M., *Compt. rend. soc. biol.*, 1949, v143, 1158.

4. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.

5. Patt, H. M., Smith, D. E., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 18.

6. Bacq, Z. M., Herve, A., Lecomte, J., and Fischer, P., *Science*, 1950, v111, 356.

7. Blount, Jr., H. C., and Smith, W. W., *Science*, 1949, v109, 83.

8. Haley, T. J., Mann, S., and Dowdy, A. H., *Science*, 1950, v112, 333.

Received February 2, 1951. P.S.E.B.M., 1951, v76.

Chemical Constituents of Skeletal Muscle from Normal Subjects and Patients with Rheumatic and Non-Rheumatic Diseases.* (18585)

EDWARD J. BIEN, MORRIS ZIFF, AND JOSEPH J. BUNIM. (Introduced by Milton Levy)
(With the technical assistance of Harold Kadin)

From the Department of Medicine and the Study Group on Rheumatic Diseases, New York University College of Medicine, and Bellevue Hospital, New York.

In the course of an investigation(1) of the incidence of lymphocytic lesions in skeletal muscle of patients with arthritis, biopsy specimens of fresh human muscle became available for simultaneous chemical analysis. Though

---

* This investigation was aided by grants from the Webster Underhill Fund and the Masonic Foundation for Medical Research and Human Welfare.

1. Sokoloff, L., Wilens, S. L., Bunim, J. J., and McEwen, C., *Am. J. Med. Sc.*, 1950, v219, 174.

the protein composition of normal and atrophic rabbit muscle has been studied in detail by Fischer and Ramsey(2), and the collagen and elastin content of normal human muscle has been determined by Lowry *et al.* (3), few data are available at present on the protein composition of human skeletal muscle and its variation in disease states. In the present study, the concentration of a number of protein constituents of human muscle were determined in normal subjects and patients with certain diseases. The chemical data obtained were correlated with the results of histological examination of the samples analyzed. A brief, preliminary report of results has been made elsewhere(4).

Methods. Muscle samples from 40 individuals were selected at random from the 202 included in the report by Sokoloff, Wilens, Bunim and McEwen(1), with the exception that individuals with a marked degree of muscular atrophy were not included because of the practical difficulty of obtaining sufficient muscle for both chemical and histological study. Muscle samples from all the 13 normal healthy adult males of the larger series were included. Each of these volunteers was thoroughly examined and it was established that they were free of any demonstrable disease. Ten were white and 3 were negroes; their ages ranged from 22 to 55. The distribution of patients according to diseases is given in Table I.

TABLE I. Distribution of Patients According to Disease.

Disease	No. of patients
Rheumatoid arthritis	8
Gout	4
Degenerative joint disease	3
Tuberculous spondylitis	4
Subacute bacterial endocarditis*	2
Marie-Strumpell spondylitis*	2
Pulmonary tuberculosis*	1
Erythema nodosum*	1
Bronchial asthma*	1
Syphilis*	1
Total	27

* Grouped in Table III as "Miscellaneous diseases."

The following muscle constituents were determined: total protein, non-protein nitrogen, collagen, myosin,† adenosinetriphosphatase (ATP-ase) activity of the myosin, and water. Under local procaine anesthesia, 3 to 4 g of gastrocnemius muscle were removed and divided into 2 portions. One was fixed for histological study, the other was immediately packed in ice and finely minced. From 0.7 to 1.0 g of mince were rapidly weighed into a thick walled centrifuge tube and the myosin was extracted by a modification of the method of Greenstein and Edsall(5). The time elapsing between excision of the muscle sample and chemical extraction was kept under 25 minutes. The mince was extracted twice, for one hour each time with 8 times its volume of cold 0.5 M potassium chloride and 0.03 M sodium bicarbonate solution. The residue after extraction was kept for the collagen determination. The combined extracts, after centrifugation in the cold, were poured into 10 volumes of copper-free distilled water and the precipitate left to settle over night in the cold. The precipitated myosin was washed with water, centrifuged in a calibrated tube and redissolved in 1.0 M potassium chloride equal in volume to the packed centrifugate. This solution was centrifuged to remove insoluble impurities, the pH adjusted to 6.8, and the volume was adjusted to exactly 10 ml. Aliquots were taken for determination of ATP-ase activity and for nitrogen content by the micro-Kjeldahl procedure. Myosin concentration was calculated from the nitrogen content on the basis of the value of 16.6 percent nitrogen determined by Bailey(7). The residue remaining after the extraction of myosin was washed three times

---

2. Fischer, E. and Ramsey, V. W., *Am. J. Physiol.*, 1946, v145, 571.

3. Lowry, O. H., Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, 1941, v139, 795.

4. McEwen, C., Bunim, J. J., Bien, E. J., Wilens, S. L., and Ziff, M., *Trans. Assn. Am. Physicians*, 1949, v62, 279.

† Myosin as referred to in this paper is "classical myosin" as extracted by Greenstein and Edsall(5) and is equivalent to the actomyosin of Szent-Gyorgyi(6).

5. Greenstein, J. P., and Edsall, J. T., *J. Biol. Chem.*, 1950, v133, 397.

6. Szent-Gyorgyi, A., Chemistry of Muscular Contraction, 1947, Academic Press, N. Y.

7. Bailey, K., *Biochem. J.*, 1942, v36, 121.

TABLE II. Volumes and Concentrations of Components Used in Determination of ATP-ase Activity.

				ml		
		1	2	3	4	5
Component	Concentration	At initial conc. of myosin	At ½ conc. of myosin	ATP control	Myosin control	Reagent control
Sodium adenosinetriphosphate	.6 mg labile P/ml	.35	.35	.35	X	X
Myosin	.30 mg N/ml	.20	.10	X	.20	X
Calcium chloride	.02 M	.10	.10	.10	.10	.10
Borate buffer	.02 M	.30	.30	.30	.30	.30
Water	X	.05	.15	.25	.40	.60

with water and allowed to stand over night at room temperature in 10 times its volume of 0.1 N sodium hydroxide. The sodium hydroxide was removed and collagen was determined in the resulting residue by the method of Lowry *et al.*(3). Collagen was determined as gelatin N in the supernatant solution after autoclaving, assuming 18.0% N in collagen (8). ATP-ase activity was measured using the sodium salt of a denosinetriphosphate as substrate. This was obtained by converting the dibarium salt[‡] with sodium amberlite IR-100 resin cycled with sodium carbonate as suggested by Polis and Meyerhof(9). The sodium ATP was adjusted to a concentration of 600 μg of hydrolysable phosphorus per ml. Myosin solutions to be used for ATP-ase activity were made up to a concentration of 0.30 mg of nitrogen per ml. As shown in Table II, ATP-ase activity was measured at 2 myosin concentrations. This was necessary to insure a substrate excess for myosin of high ATP-ase activity. When on occasion, the myosin extracts were particularly high in ATP-ase activity and a linear increase of ATP-ase activity with nitrogen concentration did not prevail, the myosin extract was diluted sufficiently to assure an adequate substrate excess. The determination was carried out at pH 9.0 in 0.006 M borate buffer, and in the presence of 0.002 M calcium chloride. In Table II are given quantities of the various substances with appropriate blanks. The reaction was stopped after incubation at 37°C for 6 minutes, by the addition of 0.5 ml of 10% trichloroacetic acid to each tube. The precipitate was centrifuged, and the phosphorus content of the supernatant solution was determined by the method of Fiske and Subbarow(10). After correcting for the blanks in tubes 3, 4 and 5 (Table II), ATP-ase activity was expressed as the number of micrograms of phosphorus liberated per mg of myosin nitrogen in 6 minutes.

For the determination of water content, a 0.2 to 0.3 g sample of muscle mince was dried at 105°C for 24 hours. Repeated determinations showed that constant weight was reached under these conditions. The dried residue was digested by the Kjeldahl procedure for the estimation of total nitrogen. Non-protein nitrogen was determined on a 0.3 to 0.4 g sample of muscle mince, which was homogenized in a Potter homogenizer. The protein was precipitated with 5 ml of 50% trichloroacetic acid, and the volume was made up to 50 ml. After filtering, a suitable aliquot was taken for micro-Kjeldahl determination. The total protein value was determined by subtracting the non-protein nitrogen from the total nitrogen. By means of this procedure it was possible to eliminate from the total protein value any extraneous nitrogen introduced into the muscle by the local procaine infiltration, although special care was taken to avoid infiltration of the muscle during biopsy. The evaluation of atrophy was based on the histological appearance of the muscle

---

8. Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods, Rev. Ed., Springfield, Ill., C. C. Thomas, 1947.

[‡] Armour and Co.

9. Polis, B. D., and Meyerhof, O., *J. Biol. Chem.*, 1947, v169, 389.

10. Fiske, C. H. and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

TABLE III. Chemical Constituents of Skeletal Muscle of Normal Individuals and Patients. (Mean values ± S.D.)

Group	No. in group	No. with lesions*	No. with atrophy	% water	Total protein as % dry wt	Myosin N as % protein N	Myosin as % wet wt	Collagen N as % protein N	Collagen as % wet wt	ATP-ase† units per mg myosin N
A. Normals without lesions	6	0	0	77.3 ±1.83	73.2 ± 2.61	23.9 ±4.18	3.99 ±0.76	12.1 ±3.64	2.01 ±0.27	1437 ±149
B. Normals with doubtful lesions	3	–	0	77.9 ±0.51	70.6 ± 3.31	19.0 ±4.30	3.06 ±0.66	10.1 ±3.60	1.58 ±0.41	1419 ±446
C. Normals with definite lesions	3	3	0	78.6 ±1.16	74.9 ± 3.42	14.0‡ ±0.28	2.10§ ±0.03	10.7 ±3.93	1.72 ±0.48	1500 ±391
Rheumatoid arthritis	8	7	7	76.2 ±3.67	60.6§ ± 7.98	14.9§ ±2.64	2.13§ ±0.24	9.48 ±2.08	1.19§ ±0.32	1038§ ±374
Degenerative joint disease	3	2	3	73.5‡ ±2.31	51.0§ ±14.1	17.8 ±5.02	2.35‡ ±0.49	10.5 ±1.84	1.23§ ±0.12	794§ ± 81
Gout	4	3	4	73.9‡ ±0.84	58.3§ ± 6.97	11.9§ ±0.87	1.69§ ±0.31	11.2 ±5.33	1.53 ±0.67	681§ ±272
Tuberculous spondylitis	4	1	0	78.4 ±1.31	71.4 ± 4.05	15.7§ ±2.61	2.38§ ±0.41	10.6 ±4.50	1.57 ±0.56	881§ ± 96
Miscellaneous diseases	8	2	1	76.6 ±1.29	68.6‡ ± 5.02	17.5§ ±4.26	2.77§ ±0.79	11.4 ±2.22	1.79 ±0.16	988§ ±445

* Lymphocytic infiltrate.

† Micrograms of phosphorus liberated in 6 min. at 37°C.

‡ Probability of chance occurrence of difference between means as compared with Group A is .05 to .01.

§ Probability is <.01. P values have been corrected for small sample size.

TABLE IV. Relation of Atrophy to Composition of Skeletal Muscle. (Mean values ± S.D.).

Group	Members of group	No. of individuals	% water	NPN as % dry wt	Total protein as % dry wt	Myosin N as % protein N	Myosin as % wet wt	Collagen N as % protein N	Collagen as % wet wt	ATP-ase units per mg myosin N
I	Normals	13*	77.9 ±1.68	1.31 ±0.12	74.0 ± 3.67	21.1 ±5.19	3.43 ±0.65	11.4 ±3.46	1.67 ±0.55	1475 ±256
II	Patients without atrophy	12†	76.8 ±2.27	1.30 ±0.46	67.2‡ ± 8.79	17.1‡ ±3.76	2.66§ ±0.72	11.3 ±2.96	1.61 ±0.46	1000§ ±340
III	Patients with atrophy	14	75.6‡ ±2.98	1.20 ±0.19	60.3§ ± 7.54	14.5§ ±3.14	2.09§ ±0.33	9.98 ±2.81	1.29‡ ±0.44	881§ ±324

* One individual was not included in Table III, as amount of muscle tissue available was insufficient for satisfactory search for muscle lesions.

† One patient in this group included in Table I was not included in Tables III and IV, as chemical analysis was incomplete.

‡ Probability of chance occurrence of the difference of the means is between 0.05 and 0.01.

§ Probability is 0.01 or less. P values have been corrected for small sample size.

sections.§ The criteria for the presence of atrophy were (1) an increase in the number of sarcolemmal nuclei and (2) a diminution of the mean fiber diameter. Both these changes are observed in the presence of well developed atrophy. With a lesser degree of atrophy, the mean fiber diameter may be reduced only slightly, because of a lack of uniformity in the degree of atrophy of the various muscle fasicles, despite the increase in the number of sarcolemmal nuclei in the atrophied areas. In such cases the atrophy was characterized as mild. All patients in whom the muscle sections demonstrated mild atrophy or greater are included in the group of "patients with atrophy" (see Group III, Table IV).

---

§ The authors are indebted to Dr. L. Sokoloff for this study.

Results. 1. *Water.* The percentage of water varied significantly from the normal only in the patients with gout and degenerative joint disease (Table III) and those with atrophy (Table IV). The mean value in the normal subjects was 77.9%. Because of the relative constancy of the muscle water content, it is possible to use wet weight as a base line in the comparison of many of the analytical data.

II. *Total protein.* The concentrations of total protein, as expressed in Table III in per cent of dry weight, are sensitive to variation in the adipose fat content of the muscle. Significance may not be attached to the absolute values of the total protein for this reason. The concentrations of myosin and collagen, however, have been expressed below in per cent of the total protein. These values are independent of variation in the adipose fat. In the 13 normal volunteers (Group I, Table IV) the total protein was 74% of the dry weight. Myosin constituted 21.1% and collagen 11.4% of the total protein. Though the volunteers were carefully screened, and showed no clinical or microscopic evidence of atrophy, in 3 of the 13 volunteers, distinct lymphocytic infiltrates were observed, and in 3 others, smaller or "doubtful" lesions were found. The relationship between the presence of these lesions and muscle composition (Table IV) is discussed below.

III. *Myosin.* The myosin fraction of the total protein, (Table III), was significantly reduced in all categories of patients studied except the group with degenerative joint disease. It was also diminished in the 3 volunteers in whom definite muscle lymphocytic infiltrates were present. Whereas the mean value of the myosin fraction of the total protein in the 6 normals without lesions was 23.9%, in the 3 "normal" individuals with lesions, the corresponding figure was 14.0%. The lowest value among patients, 11.9%, was recorded in the group with gout. The myosin concentrations as per cent of wet weight were correspondingly reduced.

IV. *Collagen.* The per cent of the muscle protein present as collagen did not depart significantly from the normal in the various disease states (Table III). The range was 9.48 to 11.2% in the patient group as compared with an average of 11.4% in the normals. The absolute content of collagen, calculated on the basis of wet weight, however, was somewhat decreased in patients with rheumatoid arthritis and degenerative joint disease.

V. *Adenosinetriphosphatase.* The ATP-ase activity of the myosin extracts (Table III)

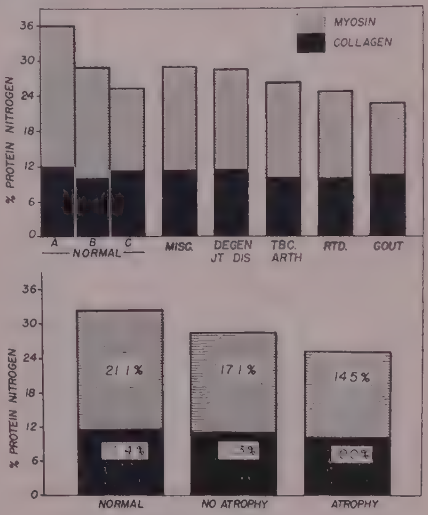


FIG. 1.

Above: Per cent of total protein nitrogen of gastrocnemius muscle as myosin and collagen. A—Subjects with no "nodules"; B—Subjects with doubtful "nodules"; C—Subjects with definite "nodules." Below: Relation between atrophy and concentration of myosin and collagen in muscle, expressed as % total protein nitrogen.

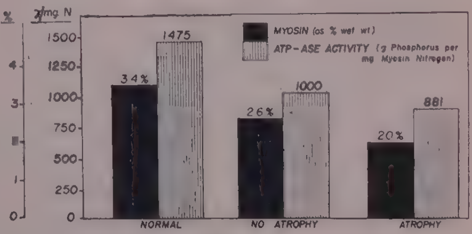


FIG. 2.

Relationship between presence of atrophy and the myosin and ATP-ase activity of gastrocnemius muscle. Myosin is expressed as % wet weight and ATP-ase activity as μg of phosphorus hydrolyzed in six minutes.

showed little variation among control subjects. It was, however, significantly reduced in all patient groups. The mean ATP-ase activity in the normals was 1437 units per mg of myosin nitrogen (Table III, Fig. 2.) In patients with rheumatoid arthritis a mean value of 1038 units was recorded. The lowest mean value, 681 units, was found in the group with gout. There was no significant statistical difference in the ATP-ase activity of the patients with miscellaneous diseases as compared with patients with rheumatic diseases. There was, however, significant difference between these two groups and the normals.

V. *Relationship of composition to muscular atrophy.* The 39 subjects were divided into 3 groups using the criteria of muscular atrophy cited above. In Group I (Table IV) were placed the 13 control subjects, in Group II the 12 patients without microscopic evidence of atrophy, and in Group III the 14 patients whose muscle sections demonstrated atrophy. Significant decreases in myosin and ATP-ase content were observed in Group II. In Group III, these changes were even more marked. Though there was a moderate fall in the wet weight per cent of collagen in the patients of Group III, the collagen fraction of the total protein was not significantly reduced in either Group II or Group III.

Discussion. It is apparent that many factors may influence the protein composition of muscle in a group of hospital patients. The age of the patient, the amount of physical activity and the nutritional status are to be considered in addition to the specific type of disease process. In selecting as experimental subjects the patients of a ward population, and comparing them with a group of active, healthy controls, it is difficult to exclude as a variable any of the above mentioned conditions. It is not surprising, therefore, that the differences in the analytical pattern noted between volunteer subjects and the patients are not specifically characteristic of any of the several incapacitating diseases studied. The changes noted, which are discussed below, appear rather to be dependent on general differences between normal individuals and the broad group of patients with musculo-skeletal disease.

Myosin, as referred to in this paper, is essentially the actomyosin of Szent-Gyorgyi (6). The determination of this protein in muscle is difficult, and the quantity extracted increases with the time of extraction. Fischer and Ramsey(2) in discussing this problem, point out that with advancement of physico chemical methods the definition of myosin has changed considerably, and that as yet "no ratio between true myosin content and myosin extractable with a certain method can be established." These authors compared the amount of myosin extracted under standard conditions with the total amount of non-collagenous protein of muscle as a base line. In the present paper, we have compared the myosin similarly extracted with the total protein content of the muscle. For the determination of myosin, we have used essentially the same extraction procedure as employed by Fischer and Ramsey, except that we have substituted potassium chloride, for lithium chloride, having found in preliminary experiments that the use of both salts gave approximately the same results. To assure more complete extraction, we have carried out two successive one-hour extractions instead of the single procedure employed by Fischer and Ramsey. The analytical figures obtained must rest in our experiments, as in those of previous investigations, essentially on an "operational definition" of the myosin. Comparison of analytical data from different individuals is based upon strictly identical procedure of extraction. The reliability of this method is indicated by the relatively good internal consistency of the analytical figures of myosin in the different groups (Table III). Comparison of these with the collagen analyses (Table III) on the same groups of individuals shows a similar spread of the analytical data in terms of standard deviation from the mean. The use of 0.4 molar alkali halide salts in the extraction of myosin has hitherto been applied only in animals and lower forms. The application of this type of extraction procedure to human muscle involves the assumption that human myosin has solubility characteristics similar to those of animal myosin. This assumption appears to be justified by the work of Bailey(7) who observed no significant differences in the properties of myosin, extracted by the method of Greenstein and Edsall(5) from a large number of animal species.

Myosin nitrogen in the 6 normal individuals without lymphocytic lesions was 23.9% of the total protein nitrogen and myosin was 3.99% of the wet weight of the muscle. The latter figure is somewhat lower than that of 4.75% observed by Fischer and Ramsey in normal rabbit muscle. The myosin content was significantly decreased in the 3 volunteers who had lymphocytic infiltrates but were otherwise normal individuals. It is difficult at this time to explain this decrease. It is important to note, however, that the ATP-ase activity of the extractable myosin from these individuals was not less than that from the other normals. Myosin was decreased significantly in all groups of patients, both as a fraction of the total protein and also as percent of wet weight. In experiments in which atrophy has been artificially induced in rabbit muscles by such measures as tenotomy and denervation, Fischer and Ramsey (2), reported decreases in myosin similar to those which we have observed in patients.

Collagen as per cent of total protein did not vary significantly between normals and patients. Though the precision of the determination of collagen is not high, the results indicate that the fraction of the total protein present as collagen was similar in both groups. It was surprising to find no relative increase in the collagen concentration in the presence of atrophy. Since neither the proteins of skeletal muscle other than myosin and collagen nor the lipids were analyzed, we cannot present data to account for the constancy of collagen concentration in the presence of falling myosin concentration. It would appear, however, that the process of atrophy in patients with musculo-skeletal disease, at least when it is mild to moderate, is not one of collagen replacement.

Beginning with the experiments of Engelhardt and Ljubimova(11) and leading up to the recent work of Szent-Gyorgyi(6), it has been shown that classical myosin (actomyosin of Szent-Gyorgyi) is intimately bound with, though separable from the enzyme ATP-ase (9,12,13). The activity of this enzyme in muscle undergoing atrophy has not as yet received attention in animal experiments. The concentration of ATP-ase in muscle may be of particular significance in evaluating the functional status of muscle because of the important role which this enzyme plays in the transformation of chemical to mechanical energy(6,7,11,14). It was, therefore, a matter of interest to study the effect of atrophy, in the course of disease, on the concentration of this enzyme system. The degree to which the ATP-activity of the myosin extracted from patients was diminished was unexpected. These findings suggest that myosin may contain a variable amount of ATP-ase from individual to individual and lends support to the point of view cited above, though not entirely accepted(9,12,13), that myosin and ATP-ase are independent substances.

Summary. 1. Gastrocnemius muscle samples from 13 normal individuals and 27 patients with arthritis and a number of miscellaneous diseases were analyzed for total protein, non-protein nitrogen, collagen, myosin, adenosinetriphosphatase and water. The chemical data were correlated with the results of histological examination of the muscle samples analyzed. 2. In muscle from 13 volunteers, myosin constituted 21.1 per cent of the total protein and 3.99 per cent of the wet weight. Significantly lower values were found, however, in all groups of patients, as well as in 3 normal volunteers in whom lymphocytic infiltrates were demonstrated microscopically. 3. The collagen fraction of the total protein did not depart significantly from the normal in any of the diseases studied, and did not change significantly in the pres-

---

11. Engelhardt, V. A., and Ljubimova, M. N., *Nature*, 1939, v144, 668.

12. Price, W. H., and Cori, C. F., *J. Biol. Chem.*, 1946, v162, 393.

13. Polis, B. D., and Meyerhof, O., *J. Biol. Chem.*, 1946, v163, 339.

14. Engelhardt, W. A., *Yale J. Biol. and Med.*, 1942, v15, 21.

ence of atrophy. The per cent of collagen based on wet weight, however, was significantly reduced in the group of 8 patients with rheumatoid arthritis and 3 patients with degenerative joint disease. 4. The adenosinetriphosphatase activity of myosin was fairly uniform among normal subjects. It was significantly reduced, however, in all categories of patients, although no statistically significant differences were found to distinguish one disease from another. 5. Atrophy was noted microscopically in 93% of a group of patients with rheumatoid arthritis, degenerative joint disease and gout. The outstanding analytical changes in the presence of muscle atrophy were decreases in the concentration of total protein, myosin and adenosine triphosphatase. There was no evidence of collagen replacement of myosin in the course of atrophy.

Received February 7, 1951. P.S.E.B.M., 1951, v76.

Electron Microscopy of Crystalline Plates in Rabbit Papilloma. (18586)

ARNOLD W. PRATT AND HERBERT KAHLER. (Introduced by H. B. Andervont)
From the National Cancer Institute, National Institutes of Health, Bethesda, Md.

Infectious papillomatosis of rabbits is characterized by warty growths of the skin (1). These lesions, of which a viral agent is considered to be the etiological agent, have been shown to have many features common to mammalian tumors(2-7). The wart arises from the basal cell layer and the growth is primarily of epithelial elements. There is extensive keratinization without accompanying desquamation. Bryan and Beard describe the lesion as consisting mostly of cutaneous horn and being a "structureless, lifeless mass of cell debris composed of pigment, keratin and insoluble, denatured protein(8)." There is a marked difference in the macroscopic appearance of the warts in domestic and wild rabbits, but Hurst has stated that there is no difference macroscopically in warts experimentally induced in domestic and wild rabbits (1a).

The present investigation reports the study of a component found in extracts of the papillomatous lesions.

Methods. The papillomatous lesions used in this study were freshly harvested from the diseased animals or were harvested previously and stored in glycerol (50%). All material, which consisted of the top half of the cutaneous cap, was carefully cleaned of hair and other superficial matter before using. The glycerol stored lesions were washed in running tap water for 2 to 6 hours to remove the excess glycerol. Homogenates of the papillomas, both from the wild and domestic rabbits, were prepared using distilled water and the Waring blendor and care was exercised to avoid heating the preparation above room temperature during this procedure. Clearing was done in a refrigerated centrifuge at 20,000 times gravity for 2 minutes. The supernatant liquid was divided into 2 portions. One portion was dialyzed against water in the cold and then examined in the electron microscope. The second portion was first examined in the electron microscope and then acetate buffer (pH 4.5) was added to bring the pH near the isoelectric point of most proteins. After another centrifugation at 20,000 times gravity for 2 minutes, a small pellet was discarded and the acetate ions

---

1. Shope, R. E., *J. Exp. Med.*, 1933, v58, 607.
2. Rous, P. and Beard, J. W., *J. Exp. Med.*, 1934, v60, 741.
3. ————, *Ibid*, 1934, v60, 701.
4. ————, PROC. SOC. EXP. BIOL. AND MED., 1935, v32, 578.
5. ————, *J. Exp. Med.*, 1935, v62, 523.
6. Syverton, J. T., Dascomb, H. E., Koomen, J., Wells, J., Berry, G. P., *Cancer Research*, 1950, v10, 379.
7. Beard, J. S., *J. Immunol.*, 1948, v58, 49.
8. Bryan, W. R. and Beard, J. W., *J. Nat. Cancer Inst.*, 1941, v1, 607.

1a. Hurst, W. E., in Shope, R. E., *J. Exp. Med.*, 1933, v58, 607.

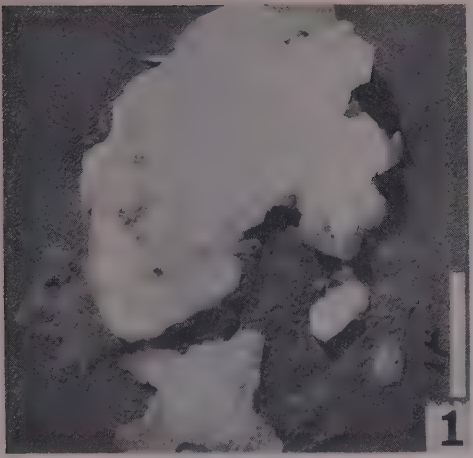


FIG. 1.
Common finding, showing the association between crystalline and non-crystalline material. Cr shadowed.

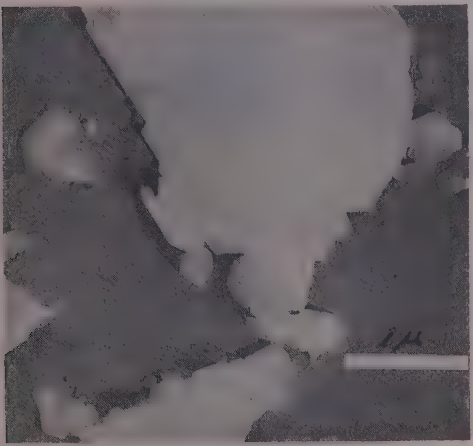


FIG. 2.
Crystals exhibiting large size range. Large opaque crystalline sheet is a crystal aggregate. Note difference in crystal thickness as shown by shadow lengths. Cr shadowed.

removed by dialyzing against water in the cold for 24 hours. The liquid was reexamined in the electron microscope. Homogenates were also prepared in an alcohol-water mixture (1:1). The supernatant fluid of these preparations was centrifuged and the sedimented pellet resuspended in water for examination in the electron microscope. Acetone and chloroform were added to the supernatant of the water homogenates and repeatedly shaken together. After separation of the water and acetone or the water and chloroform had occurred, samples were taken from the water fraction for examination.

Results. Crystalline material, like that shown in the accompanying micrographs is always found in the water extracts of the papillomatous lesions. It is present in the freshly harvested and glycerin stored warts. The close association of crystalline material and non-crystalline material, which is quite characteristic, can be seen in Fig. 1. Treatment of the preparations with acetate buffer appeared to effect a partial separation of the crystalline material and the non-crystalline substances.

The individual crystals which we have observed can be divided roughly into single and multiple crystals. The single crystals are represented by the very small, hexagonal plates seen in Fig. 2. The multiple crystals appear to be made of several of the hexagonal plates crystallized together as represented in Fig. 3 and 4. The large multiple crystals frequently have the property of being laminated structures made up of thin crystalline sheets which can be noted in Fig. 3 and 4. These crystalline sheets show a wide variation in surface area but usually exhibit a remarkable degree of thinness. The smaller crystals measure approximately 125 mμ over their shortest distance while the large crystals measure several microns. Of especial interest are the small hexagonal plates seen on and between the crystalline sheets. All measurable angles of the crystals, single or multiple, are approximately 120 degrees.

The crystals exhibit no change in shape, size or density under the very hot electron beam of the bias gun. If they are first viewed in the electron microscope (for identification) then the screen removed and washed with water for a period of time, there is no change in the morphology of the crystals. However, if a water homogenate is permitted to stand for several hours, either at room temperature or in the cold, the crystals will break up into smaller entities and ultimately disappear suggesting that they are somewhat soluble in water. After the crystals once disappear on standing, they do not reform suggesting that their occurrence is not an artifact of drying

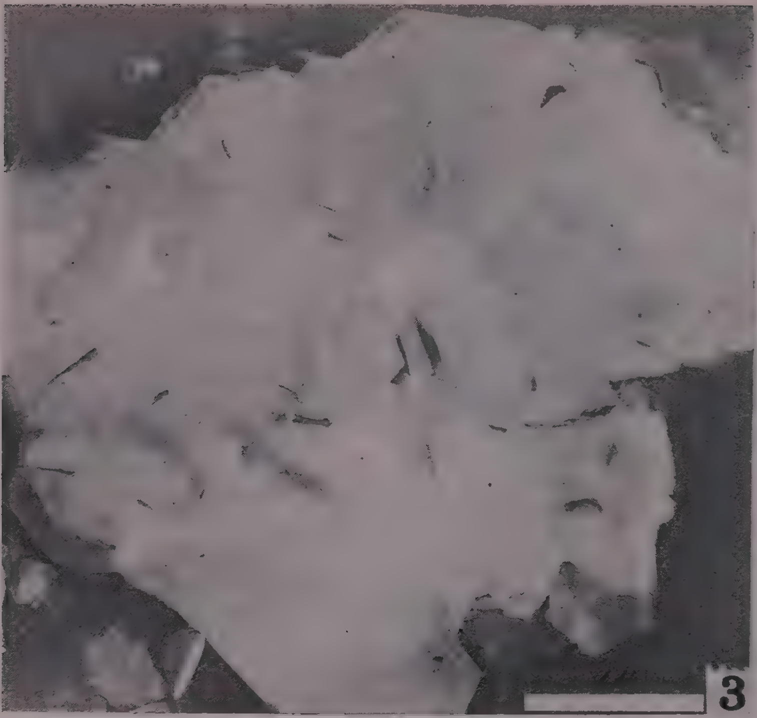


FIG. 3.
Large lamellar sheets of predominantly crystalline material. Cr shadowed.

and presumably that they form during the development of the papillomatous lesion. Examination of the water solutions containing crystals to which acetone, chloroform or alcohol had been added by the stated procedures continued to show crystals suggesting the lack of solubility in these organic solvents. We have not yet separated the crystals with any degree of purity from the contaminating matter which is always found in an extract of this kind. The extracts which had been partially cleared of protein by centrifugation in acetate buffer and then dialyzed were subjected to ultracentrifugal fractionation, light absorption studies, electron diffraction and electrophoresis. No positive identification was made.

Discussion. It appears from our studies that a component of the papillomatous lesions exists in the crystalline state. The finding of the crystals in both fresh and glycerol stored papillomas excludes the possible dessicating action of the glycerin forming the crystals during storage. They are only sparingly soluble in water thus making their presence in the water extracts indicative of their existence in the papilloma in a crystalline state. These crystals, in contrast to the recently observed virus crystals, are more like the classical crystalline aggregates and cannot be resolved into discrete macromolecular components in the electron microscope(9).

A review of the literature which discusses the histopathology of the papillomas suggests a mechanism whereby the crystals could be formed. It is known that the virus causes a lively proliferation of the basal cell layer of the epithelium. The growing epithelium first extends under the adjacent epithelium, and downwards to the fibrous corium. thereafter protruding upwards as papillae which may grow to a large size. There is definite inter-

---

9. Wyckoff, R. W. G., *Interscience Publishers, Inc.*, New York, pp. 151-178.

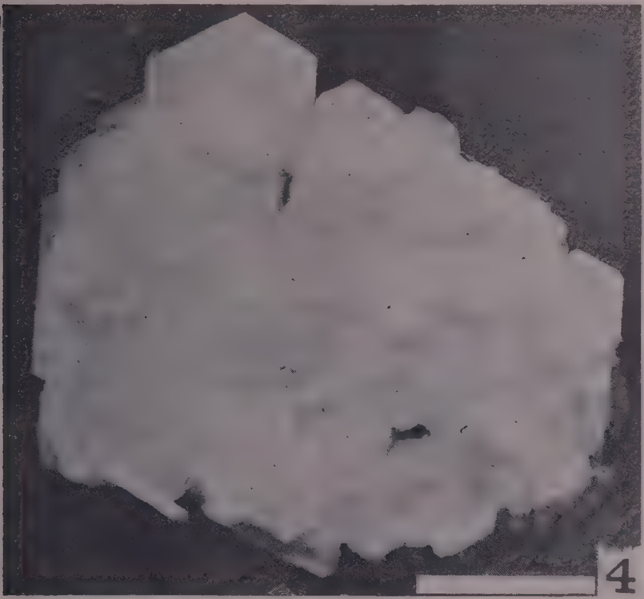


FIG. 4.
Large crystalline aggregate showing the laminated structure and many adherent smaller crystals. Note the tendency for the orientation of the crystals to be affected by their neighbors. Cr shadowed.

ference with the blood supply because of the lateral pressure with resultant drying of the tops of the papillae. Desquamation does not occur so that the growth becomes capped with a firm, dessicated cutaneous horn(3). If the conditions of drying and internal pressure are sufficient then crystallization may occur. The formation of such discrete crystals means that there must be available in a local region and in some concentration, a substance which can undergo crystallization. Whether these crystals are formed by a lytic action during degeneration or whether it is a normal substance produced during the growth process is not known.

Summary. The finding of crystalline platelets in the water extracts of papillomatous lesions of the wild and domestic rabbit is noted. A few of the elementary properties of the crystals are discussed.

Received February 13, 1951. P.S.E.B.M., 1951, v76.

Agglutination of Red Cells by Synthetic Lysine Polypeptides.* (18587)

J. R. RUBINI, M. A. STAHMANN, AND A. F. RASMUSSEN, JR.
From the Departments of Biochemistry and Medical Microbiology, University of Wisconsin.

It has been shown that basic high molecular weight lysine polypeptides(1), are capable of aggregating tobacco mosaic virus(2), and ag-

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a research grant from the National Institute of Health, Public Health Service and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1. Becker, R. R., and Stahmann, M. A., *J. Am Chem. Soc.*, in press.

TABLE I. Hemagglutination by Synthetic Lysine Polypeptides.

Polypeptide	Amino acid residues per molecule*	Molecular wt*	Hemagglutination activity,† μg/ml
L-lysine polypeptide	6	980	7.8
” ”	18	3000	0.48
” ”	85	14000	0.24
” ”	158	26000	0.12
DL-lysine, DL-valine polypeptide	25	4000	3.9
DL-lysine polypeptide	36	6000	1.9

* The number of amino acid residues per molecule and the molecular weights were determined by Van Slyke amino nitrogen analysis of the terminal amino acid groups and represents an average figure(1).

† Smallest amount causing hemagglutination.

glutinating certain bacteria(3). In this investigation, the ability of a number of the synthetic lysine polypeptides to agglutinate red blood cells was studied. The polypeptides were found to agglutinate erythrocytes at concentrations as low as 0.1 μg of polypeptide per ml. When the peptides were injected into embryonated eggs, it was found that the allantoic fluid contained enzymes capable of destroying the compounds. The agglutination of erythrocytes and this enzymatic destruction of the polypeptide by allantoic fluid are reported here.

Materials and methods. The various synthetic lysine polypeptide hydrochlorides (Table I) were dissolved in (physiological) saline to concentrations of 10 mg per ml; the solutions were adjusted to pH 7.0-7.2 and kept at 4°C. Aliquots were removed and diluted with saline to the desired concentrations as needed. The hemagglutination titrations of the polypeptides were carried out in plastic plates using the pattern end point(4,5). To 0.5 ml of serial 2-fold dilutions of the polypeptide solution was added 0.25 ml of a 1% suspension of washed chicken red cells. The plates were shaken to mix the contents; and after standing approximately 2 hours, the endpoints were read as the highest dilution showing partial or complete hemagglutination. The endpoints were found to be reproducible to within one dilution. The allantoic fluids were obtained from fertile eggs on the 12th day of incubation. The sterile fluids were pooled and cleared by centrifugation at low speeds before use.

Experimental. Various polypeptides were titrated for their hemagglutination endpoints. Table I shows the hemagglutination titers. It is seen that the titers of the L-lysine polypeptides are related to their chain lengths; the 36 unit DL-lysine and the 25 unit DL-lysine-DL-valine polypeptides were less active when compared to the L-lysine series.

Solutions of the amino acids L-lysine, DL-lysine, DL-valine, L-histidine, and L-glutamic acid were tested for hemagglutination. No agglutination was produced by any of the amino acids. The highest level contained 80,000 μg amino acid per ml. This was almost 1 million times the concentration of the 158 unit L-lysine polypeptide at its endpoint.

To test the possibility that the amino acids would inhibit polypeptide hemagglutination, solutions containing 1% red cells and various concentrations of the amino acids were used to titer the 85 unit L-lysine and 36 unit DL-lysine polypeptides. No hemagglutination inhibition was detected although amino acid concentrations as high as 40,000 μg per ml were tested.

It was of interest to determine whether red cells, agglutinated by the polypeptides, would lyse in the presence of complement. Experiments were carried out in which the 85 unit L-lysine polypeptide was tested for lytic properties. It was found to be unable to lyse red cells in the presence of complement. Hemolysin-sensitized sheep cells were agglutinated by concentrations of 100, 10, 1 and 0.1 μg per ml of the 85 unit polypeptide, and sufficient complement was added to lyse the controls. Within an hour, complete lysis occurred in mixtures containing no added polypeptide and 0.1, and 1.0 μg polypeptide per ml; after approximately 8 hours, the cells agglutinated by

---

2. Burger, W. C., Master of Science Thesis, Biochemistry Department, University of Wisconsin, 1950.
3. Stahmann, M. A., Burger, W. C., Sill, W., and Walker, J. C., unpublished results.
4. Hirst, G. K., *J. Exp. Med.*, 1948, v87, 301.
5. Hirst, S. A., *Science,* 1948, v108, 479.

TABLE II. Destruction of the 85 Unit L-Lysine and 36 Unit DL-Lysine Polypeptides by Normal Allantoic Fluid *in Vitro*.

85 unit L-lysine polypeptide			36 unit DL-lysine polypeptide	
Temp. of incubation, °C	Hemag. activity		Temp. of incubation, °C	Hemagglutination activity after 22 hr,* μg/ml
	Time, hr*	μg/ml		
4	30	0.12	4	1.6
25	30	1000†	25	1.6
37	6	1000	37	3.1
51	2	1000	51	12.5
65	4	1000	70	25
100	30	0.10		
4‡	30	0.12	4‡	1.6
65‡	30	0.12	70‡	1.6

* Portions of the solutions were removed and titrated after ½, 2, 4, 6, 8, 12, 14, 24, and 30 hours incubation.

† The undiluted test solutions, containing 1000 μg polypeptide per ml, did not agglutinate red cells.

‡ Control, in saline.

10 μg per ml of the polypeptide lysed; but 100 μg of polypeptide per ml prevented lysis and the cells remained strongly agglutinated.

Hirst(4) has shown that treating fowl red cells with periodate destroys their ability to adsorb influenza and other related viruses. Using Hirst's method, periodate treated cells were prepared. These cells which were unable to take up influenza virus, readily adsorbed the 85 unit L-lysine polypeptide in amounts equal to untreated red cell controls. This indicates that virus receptors are not necessary for agglutination by the polypeptide.

When allantoic fluid from eggs which had survived injection of large amounts of lysine polypeptides were titrated for residual polypeptide, no hemagglutination was observed. However, when the 85 unit L-lysine polypeptide was serially diluted in allantoic fluid and titrated immediately, the endpoint was the same as that obtained by serially diluting the polypeptide in saline. This showed that there was no polypeptide hemagglutination inhibitor in the allantoic fluid. Rather these observations suggested a destruction or removal of the polypeptide *in vivo* in the egg. *In vitro* experiments in which the polypeptide was incubated with pooled allantoic fluid at various temperatures indicated destruction of the L-polypeptide. That this destruction was an enzymatic destruction of the polypeptide is suggested by the data shown in Table II. These data show that at 4° there is no destruction of the L-85 unit polypeptide, but rapid destruction occurs above 25°. Similar experiments in which the 36 unit DL-lysine polypeptide replaced the L-isomer showed only very slight destruction. When the L-polypeptide was incubated for 24 hours with allantoic fluid which had previously been heated to 100° there was no loss of activity while fluids which had previously been heated to 65° retained the capacity to inactivate completely the hemagglutinating activity of the polypeptide.

Discussion. The hemagglutination titers of the various synthetic lysine polypeptides appear to be correlated with the average number and type of amino acid residues per molecule. It is seen here that the longer L-lysine polypeptides are more effective in hemagglutination in low concentration than the shorter L-lysine polypeptides. The reduced endpoint of the DL-polypeptide as compared to the L-polypeptides shows that the L-lysine configuration is most effective. The DL-valine-DL-lysine polypeptide also showed a reduced activity.

The complete absence of agglutination by high concentration of lysine and other amino acids and the increase in activity as the molecular size of the lysine polypeptides is increased indicate that the lysine residues must be joined together to cause hemagglutination. It is postulated that hemagglutination occurs when the basic polypeptide collides with the surface of the red cell and forms many ionic bonds between basic butyl amino side chains of the peptide and acidic groups at the surface of the cell. Then as these polypeptide-cell complexes which now have a reduced electrical repulsion for other cells collide, more ionic bonds are formed between the polypeptide and the second cells. Thus, the polypeptide serves to link togther the red cells, and by repetition of this process the agglutinated cell masses are formed. Although the bond strength of any one individual ionic bond that is formed in such a process is relatively weak, the formation of many such bonds between the peptide molecule and the red cells stabilizes the complex. Disassociation is greatly reduced compared to that with the free amino acids because the peptide chain holds the positively charged groups of the peptide together and close to the red cell surface to form regions of very high ionic concentration which by mass action favors the combination or recombination with the negatively charged groups at the cell surface.

The 85 unit L-lysine polypeptide was shown to inhibit lysis of sensitized sheep cells upon the addition of complement. It appears likely that at high concentrations, the polypeptide molecules had sufficiently coated the cell surfaces so that complement was unable to cause lysis. Previously it had been shown(6) that the rate of formation of a precipitate of tobacco mosaic virus with its anti-serum was affected by the addition of small amounts of an L-lysine polypeptide. These two phenomena are both believed to result from the ability of the polypeptide molecules to coat acidic surfaces.

Abderhalden(7) had found that proteolytic enzymes were present in the chick embryo after the 7th day of incubation. Evidence was here presented that the synthetic lysine polypeptides were destroyed by enzymes in the allantoic fluid. The L-lysine polypeptides were hydrolyzed *in vitro* by the allantoic fluid at a rapid rate, whereas the DL-polypeptide showed only slight hydrolysis. The enzymes in the fluid could be inactivated by heating the fluids to 100°C.

Summary. Basic, high-molecular weight synthetic lysine polypeptides have been found to agglutinate chicken red cells at concentrations as low as 0.1 μg per ml. The hemagglutination titer of the polypeptides appears to be related to the number and type of amino acid residues per molecule. The polypeptides are enzymatically hydrolyzed by normal allantoic fluid of chicken embryos.

---

6. Stahmann, M. A., and Mathews, R. E. F., unpublished results.

7. Abderhalden, E., and Steinbeck, E., *Z. Physiol. Chem.*, 1910, v68, 312.

Received February 13, 1951. P.S.E.B.M., 1951, v76.

Inhibitory Effect of Synthetic Lysine Polypeptides on Growth of Influenza Virus in Embryonated Eggs.* (18588)

J. R. RUBINI, A. F. RASMUSSEN, JR., AND M. A. STAHMANN.

From the Departments of Biochemistry and Medical Microbiology, University of Wisconsin, Madison.

Studies on some of the biological properties of a group of basic, high molecular weight, lysine polypeptides recently synthesized in this laboratory(1) revealed them to be capable of combining through ionic linkages with acidic groups at the surface of tobacco mosaic

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a research grant from the National Institute of Health, Public Health Service and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1. Becker, R. R., and Stahmann, M. A., *J. Am. Chem. Soc.*, in press.

TABLE I. Synthetic Lysine Polypeptides.

Polypeptide hydrochloride	Amino acid residues per molecule*	Molecular wt*	Max. tolerated dose in mg/0.1 ml
L-lysine polypeptide	6	980	2.0†
,, ,,	18	3000	1.0
,, ,,	85	14000	.1
,, ,,	158	26000	.2
DL-lysine polypeptide	36	6000	.2
,, ,,	240	40000	.1†
DL-lysine, DL-valine polypeptide (Copolymer)	25	4000	.5

* The number of amino acid residues per molecule and the molecular weights were determined by Van Slyke amino nitrogen analyses of the terminal amino end groups and represent an average figure(1).

† Highest levels tested; the maximum tolerated dose was not determined.

virus. The L-lysine polypeptides formed relatively insoluble complexes with the virus, whose infectivity was thereby markedly reduced(2). Many animal viruses have also been found to have acidic surfaces which allow their combination with basic materials(3). In the present study, a group of basic, synthetic lysine polypeptides were screened for their ability to inhibit the growth of influenza virus in the embryonated egg.

Materials and methods. Stock solutions of the various lysine polypeptides (Table I) were prepared by dissolving their hydrochloride salts in (physiological) saline to a concentration of 10 mg polypeptide per ml. The solutions were adjusted to pH 7.0 to 7.2 and stored at 4°C until needed, when portions were removed and diluted with saline to the desired concentrations.

The influenza virus used was from a stock of frozen allantoic fluids from eggs infected with influenza virus, type A, PR8 strain. Fertile eggs previously incubated for 11 or 12 days were employed throughout in the experiments. The method used for the virus hemagglutination titrations was that of Salk(4,5) employing plastic plates. Serial 2-fold dilutions in saline of virus infected allantoic fluids were made in 0.5 ml amounts using plastic serological plates. One-fourth ml of a 1% suspension of washed chicken red cells was then added to each serial dilution of the fluids. The plates were shaken to mix the fluids and after approximately 2 hours, the end points were read as the highest dilution showing complete or partial hemagglutination.

Experimental. The toxicities of the various polypeptides were determined by injecting graded doses in volumes of 0.1 ml into the allantoic cavities of groups of 10 eggs. After 24 hours incubation, the eggs were candled to determine the number of survivors. The maximum non-lethal doses (MNLD) of the polypeptides are shown in Table I.

Varying doses of the different polypeptides were injected into the allantoic cavities of small groups of eggs. One hundred thousand minimum infective doses (MID_{50}) of the virus were injected either with, before, or after the polypeptides in a volume of 0.1 ml. After incubation for 24 hours, portions of the fluids were removed and titrated individually. Averages of each group were calculated and compared to an equal or larger number of controls inoculated with the virus alone. It is generally recognized that the infectivity of allantoic fluid containing viable virus is roughly proportional to the hemagglutination titer of that fluid. It was believed that the hemagglutination titers measured the amounts of virus with sufficient accuracy for this preliminary work. It was shown(6) that under these experimental conditions the polypeptides are enzymatically destroyed *in vivo* within 24 hours so that they did not interfere with the virus hemagglutination titers. The polypeptides were also tested against smaller virus inocula for longer incubation periods. In some cases the polypeptides were injected initially and again 24 hours later.

---

2. Burger, W. C., Master of Science Thesis, Biochemistry Department, University of Wisconsin, 1950.

3. Warren, J., Weil, M. L., Russ, S. B., and Jeffries, H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 662.

4. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

5. Salk, J. E., *Science*, 1948, v108, 479.

6. Rubini, J. R., Stahmann, M. A., and Rasmussen, A. F., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 659.

TABLE II. Allantoic Fluid Hemagglutination Titers 24 Hours After the Virus Inoculation.

Polypeptide	Dosage in mg/0.1 ml	Survival / Total treated	Avg titer of Treated	Avg titer of Control
A. Polypeptides inj. 1 hr before 100000 MID$_{50}$ of virus.				
6 unit L-lysine	2.0*	4/4	640	1280
6 " "	1.0*	4/4	640	1280
18 " "	1.0	2/4	160	1280
18 " "	.1	4/4	640	1280
85 " "	.1	2/4	160	1280
158 " "	.2	4/6	160	1280
158 " "	.1	8/10	80	1280
36 " DL-lysine	.2	7/16	160	1280
36 " "	.1	4/4	10	1280
240 " "	.1	8/9	640	2560§
240 " "	.05	8/9	640	2560§
25 " " DL-valine	.5	5/6	320	1280
25 " " "	.2	6/6	320	1028
B. Polypeptides inj. together with 100000 MID$_{50}$ of virus.				
85 unit L-lysine	.1	5/6	640	5120†
36 " DL-lysine	.2	10/15	320	1280
240 " "	.1	9/9	640	2560§
240 " "	.05	9/9	80	2560§
25 " " DL-valine	.5	14/18	10	640
C. Polypeptides inj. 3.5 hr after 100000 MID$_{50}$ virus.				
36 unit DL-lysine	.1	7/8	160	640
25 " " DL-valine	.5	6/8	160	640
25 " " "	.5‡	9/12	80	1280

* 15 min. before virus.
† Titer at 30 hr.
‡ 15 min. after virus.
§ The 240 DL-lysine unit titers are of pooled fluids.

Results. The results of representative experiments are shown in Table II. Generally, it was found that the degree of inhibition as measured 24 hours after inoculation increased with the dosage of polypeptide. There was a correlation between the degree of inhibition and the average number of amino acid residues per molecule of the L-lysine polypeptides. With one exception, the greatest inhibition was shown when the polypeptides preceded the virus. The one exception was the 25 unit DL-lysine-DL-valine polypeptide which, when injected with the virus, prevented the development of hemagglutinins within 24 hours in every case.

When the amounts of virus were measured 48 hours or longer after inoculation and treatment with the polypeptides no significant inhibition was obtained even against smaller virus inocula of 1,000 MID$_{50}$. Repeated doses of the polypeptides initially and 24 hours after the inoculation of virus also failed to prevent the appearance of hemagglutinins by 48 hours.

Discussion. The toxicities of the various lysine polypeptides are seen to be related to the average number and type of amino acid residues per molecule. However, the very long polypeptide chains, the L-158 unit and the DL-240 unit lysine polypeptide, appear to be somewhat less toxic than the L-85 unit lysine polypeptide. It appears likely that the toxic effect of the polypeptides is caused by a combination of the basic peptides with essential acidic groups.

The L-158 unit, DL-36 unit, DL-240 unit lysine polypeptides and the 25 unit DL-lysine-DL-valine polypeptides significantly delayed the production of hemagglutinins even when very large inocula containing 100,000 MID$_{50}$ of virus were employed. The data obtained in these experiments suggest that the basic polypeptides combine directly with the virus particles to inhibit infectivity. Inhibition was evident when the polypeptides were injected before or with the virus. When the polypeptides were injected after the virus, less inhibition was obtained. The 25 unit lysine-valine polypeptide was most effective when it was injected with the virus;

when injected 15 minutes before or after the virus, less inhibition was observed. The fact that no inhibition of virus growth was demonstrable after 48 hours incubation further suggests that the polypeptides combine with the virus but active virus is subsequently released.

The inhibition of tobacco mosaic virus infectivity(2) and this inhibition of the production of influenza virus hemagglutinins by the lysine polypeptides suggest that these or similar synthetic polypeptides might be active against other viruses. The increased activities of the DL-lysine polypeptide and of the DL-lysine-DL-valine polypeptide suggest that the stereochemical structure as well as the amino acid composition of such synthetic polypeptides might be varied in order to produce greater antiviral activity.

Preliminary experiments failed to show a significant chemotherapeutic effect against influenza virus in mice when the polypeptides were administered as an aerosol.

Summary. Various synthetic high molecular weight lysine polypeptides were found to inhibit the growth of influenza virus in the chick embryo.

Received February 13, 1951. P.S.E.B.M., 1951, v76.

Circulating Antibodies in Vitamin-Deficiency States. Pantothenic Acid and Pyridoxine Deficiencies.* (18589)

Peter P. Ludovici, A. E. Axelrod,† and Bettina B. Carter.
(Introduced by Ralph R. Mellon)

From the Institute of Pathology, The Western Pennsylvania Hospital, and the Department of Chemistry, University of Pittsburgh.

A severe impairment of antibody response has been observed in pyridoxine(1,2) and pantothenic acid-deficient rats(1). Riboflavin, biotin, and thiamine deficiencies had considerably less effect(1,3,4). In our later experiments(5), particular emphasis was placed upon the use of "paired-weighed" in lieu of "paired-fed" rats as controls to the pantothenic acid-deficient animals. The evidence obtained proved conclusively that the absence of pantothenic acid *per se* and not the concomitant inanition was responsible for the decreased serum antibody titers. In addition, the pronounced effects of a relatively mild pantothenic acid deficiency were also demonstrated. It is generally recognized that a decreased antibody titer in serum is not unequivocal proof for the actual impairment of antibody synthesis. Other factors such as the effects of the vitamin deficiency upon the processes of antibody destruction and distribution must be evaluated before a definite relationship between vitamin and antibody synthesis can be established. Thus, a dietary inadequacy of pantothenic acid or pyridoxine may result in: (1) an actual impairment of antibody synthesis; (2) an accelerated rate of antibody destruction; or (3) a failure to release and distribute antibodies from their site of formation into the blood stream. Any one of these three mechanisms could produce a lowered serum antibody concentration.

The present experiments were, therefore, undertaken in an effort to shed some light on

---

* This investigation was supported by grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, the National Vitamin Foundation, Inc., and Hoffman-La Roche, Inc.

† Present address: Institute of Pathology, Western Reserve University, Cleveland, O.

1. Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R., Proc. Soc. Exp. Biol. and Med., 1947, v66, 137.

2. Stoerk, H. C., and Eisen, H. N., *Ibid.*, 1946, v62, 88.

3. Carter, B. B., and Axelrod, A. E., *Ibid.*, 1948, v67, 416.

4. Stoerk, H. C., Eisen, H. N., and John H. M., *J. Exp. Med.*, 1947, v85, 365.

5. Ludovici, P. P., Axelrod, A. E., and Carter, B.B., Proc. Soc. Exp. Biol. and Med., 1949, v72, 81.

TABLE I. Effect of Pantothenic Acid Deficiency Upon Serum Content of Preformed Antibody.*

Rat No.	Weeks on experiment after the initial bleeding						
	0	2	4	7	10	13	18
Group I†							
1	1:640	1:640	1:2560	1:2560	1:2560	—	—
2	1:320	1:640	1:1280	1:2560	1:1280	1:2560	1:1280
3	1:320	1:320	1:2560	1:1280	1:1280	1:2560	1:1280
4	1:160	1:640	1:1280	1:2560	1:2560	1:640	1:2560
Group II†							
5	1:640	1:640	1:2560	1:2560	1:2560	—	—
6	1:320	1:320	1:2560	1:2560	1:2560	1:2560	1:1280
7	1:160	1:640	1:2560	1:1280	1:1280	1:2560	1:640
8	1:320	1:320	1:640	1:2560	1:2560	1:2560	1:2560

* Individual hemagglutinin titers are presented.

† Group I, inanition control animals No. 1, 2, 3, and 4 were "paired-weighed" with Group II, pantothenic acid-deficient rats No. 5, 6, 7, and 8, respectively.

the mechanisms which may be operative in the impaired response of pantothenic acid and pyridoxine-deficient rats to the antigenic stimulus of human erythrocytes.

Methods. Male weanling albino rats of the Sprague-Dawley strain were employed throughout this investigation. The animals were housed individually in wide-meshed, screen-bottom cages. All rats were fed a basal diet with the following percentage composition: sucrose, 56.76; Labco "vitamin free" casein, 25.00; salts(6), 4.00; cod-liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; *i*-inositol, 0.03; *p*-amino-benzoic acid, 0.01; *dl-α*-tocopherol acetate, 0.01; and 2-methyl-1,4-naphthoquinone, 0.001. All animals received additional vitamins in the form of a daily pill. Each of the pills fed to the control rats supplied the following vitamins: thiamin, 40 γ; riboflavin, 60 γ; pyridoxine, 5 or 50 γ; nicotinic acid, 100 γ; calcium pantothenate, 100 or 300 γ; biotin, 1 γ; and folic acid, 1 γ. The basal diet plus the complete vitamin pill will be referred to as the control diet in this paper. Unless otherwise specified, the complete vitamin pill contained 50 γ of pyridoxine and 300 γ of calcium pantothenate. For the pantothenic acid and pyridoxine-deficient animals, the appropriate vitamin was omitted from the pill. The procedure of "paired-weighing" consisted of restricting the amount of basal diet consumed by the control animal to a quantity sufficient to maintain its weight equal to that of its vitamin-deficient partner. Deficient rats were always fed *ad libitum.* Immunization was accomplished by injecting each rat intraperitoneally with a 10% suspension of Group O, Rh positive human erythrocytes in physiological saline. An initial dosage of 0.5 ml of the red blood cell suspension was followed by 2 one ml inoculations on alternate days. Five days after the final injection and at specified intervals thereafter the animals were bled from the heart under nembutal anesthesia.‡ The sera were tested for agglutinin titer utilizing serial two-fold dilutions as previously described(1). End point titers as observed in this laboratory are subject to an error of one dilution in either direction.

Experimental. A. *Effect of pantothenic acid or pyridoxine deficiency upon serum content of preformed antibody.* In these experiments, we investigated the possibility that the impairment of antibody response observed in pantothenic acid and pyridoxine deficiencies might be due to an increased rate of antibody destruction.

1. *Pantothenic acid deficiency.* Eight weanling rats were fed the control diet containing 100 γ of pantothenic acid *ad libitum* for 23 days. Immunization was instituted on the 14th day. After the initial antibody determinations, the results of which are shown in Column 2, Table I, the animals were paired

---

6. Jones, J. H., and Foster, C. J., *J. Nutrition,* 1942, v24, 245.

‡ Three mg per 100 g body weight were injected intraperitoneally.

according to titer into two equal groups. One group received the control diet minus pantothenic acid, while the other received the control diet containing 300 γ of pantothenic acid daily. Members of the latter group were "paired-weighed" with those in the former group. Serum antibody titers were determined periodically until the deficient rats succumbed. Following transfer to the deficient diet, the animals (average weight at the initial bleeding = 185 g) gained weight slowly until a maximum average weight of 300 g was attained after approximately 12 weeks on this regimen. Thereafter, the rats gradually lost weight until the experiment was terminated at 18 weeks, (final average weight = 240 g). One deficient rat succumbed during the 12th week while the remaining three were moribund at the final bleeding. Typical pantothenic acid deficiency symptoms were observed in these animals approximately one month prior to death. The "paired-weighed" controls, although severely starved, survived the experimental period. It may be seen in Table I that during the course of the experiment there was no significant difference between the antibody titers of the pantothenic acid-deficient rats and those of their inanition controls. It is of interest to note that relatively high antibody titers were demonstrable 18 weeks after immunization. We have similarly observed that normal rats fed the control diet *ad libitum* exhibited high antibody levels seven months after immunization (unpublished observations). It can be further seen in Table I that the initial titers were relatively low increasing to normal levels 4 weeks after the initial bleeding. A similar influence of age upon antibody formation in *young* normal rats has been observed frequently in this laboratory. Freund(7) has made comparable observations in rabbits.

TABLE II. Effect of Pyridoxine Deficiency Upon the Serum Content of Preformed Antibody.*

Rat No.	Weeks on experiment†			
	0	4	8	12
	Group I‡			
1	1:640	1:2560	1:2560	1:2560
2	1:2560	1:2560	1:2560	1:2560
3	1:2560	1:2560	1:2560	1:2560
4	1:2560	1:2560	1:2560	1:2560
5	1:2560	1:2560	1:1280	1:1280
	Group II‡			
6	1:2560	1:2560	1:2560	1:1280
7	1:2560	1:2560	1:1280	1:640
8	1:2560	1:2560	1:1280	1:640
9	1:2560	1:2560	1:2560	1:2560
10	1:2560	1:2560	1:2560	1:1280

* Individual hemagglutinin titers are presented.

† Weeks after the initial bleeding.

‡ Group I, inanition control animals No. 1, 2, 3, 4, and 5 were "paired-weighed" with Group II, pyridoxine-deficient rats No. 6, 7, 8, 9, and 10, respectively.

2. *Pyridoxine deficiency*. A similar experiment to determine the effect of a pyridoxine deficiency upon preformed antibody was conducted. Ten weanling rats were fed the control diet containing 5 γ of pyridoxine *ad libitum* for 31 days. Immunization was instituted on the 22nd day of the experiment. Following the initial bleeding, the animals were paired according to weight into two equal groups. "Paired-weighed" controls received the control diet containing 50 γ of pyridoxine daily, whereas pyridoxine was omitted from the diet of the remaining animals. Blood samples for antibody titration were taken periodically until the experiment was terminated.

Following the removal of pyridoxine from the diet, the rats (average weight at the initial bleeding = 145 g) continued to gain weight slowly until a maximum average weight of 200 g was attained after approximately 10 weeks. Thereafter, the animals gradually lost weight until the experiment was terminated at 12 weeks, (final average weight = 190 g). Incipient symptoms of pyridoxine deficiency became apparent during the 9th week and were well developed by the 12th. All deficient rats as well as their inanition controls survived the experimental period. It may be seen in Table II that there was no significant difference between the antibody titers of the pyridoxine-deficient rats and those of their inanition controls during the three month period. These results were comparable to those observed in the previous experiment where pantothenic acid was removed from the diet (Table I).

B. *Effect of adrenal cortical extract or*

---

7. Freund, J., *J. Immunol.*, 1930, v18, 315.

vitamin therapy upon serum antibody levels of vitamin-deficient rats. These experiments were concerned with the possible role of pantothenic acid or pyridoxine in the release and distribution of antibodies from their site of formation into the blood stream.

1. *Effect of aqueous adrenal cortical extract*[§] *(A.C.E.) upon serum antibody titers of pantothenic acid-deficient rats.* Pathological changes in the adrenal cortex, as well as a probable depletion of adrenal cortical hormones, have been demonstrated in pantothenic acid-deficient rats(8-11). Since adrenal cortical activity may be concerned with the liberation of antibodies from their site of formation(12), it is conceivable that the lowered serum antibody titers of pantothenic acid-deficient rats could be attributed to a decreased activity of the adrenal cortex. This hypothesis was tested as follows: Seven weanling rats were fed *ad libitum* the control diet minus pantothenic acid. "Paired-weighed" partners of each of these rats received the control diet containing 300 γ of pantothenic acid daily throughout the experiment. Immunization was instituted in all animals after 6 weeks. Four of the 7 animals in each group were injected subcutaneously with one ml of A.C.E. immediately following each injection of the antigen. It is evident from the results presented in Table III that A.C.E. had no effect upon the antibody titers of either inanition controls or pantothenic acid-deficient rats. No influence of the extract upon the growth of pantothenic acid-deficient animals was observed. In a similar experiment except that A.C.E. was administered in a single 2 ml dose three weeks *following* immunization, the extract was again ineffective in elevating the antibody titers of 4 "paired-weighed" controls and 4 pantothenic acid-deficient animals. In this case antibody titers were determined 5 and 18 hours following A.C.E. injection. These results, therefore, furnish no support for the suggestion that the activity of pantothenic acid in antibody fabrication is mediated through the adrenal cortex.

TABLE III. Influence of Aqueous Adrenal Cortical Extract Upon Serum Antibody Titers.[*]

Group I[†] Inanition controls	Group II[†] Inanition controls + A.C.E.[‡]	Group III Pantothenic acid-deficient	Group IV Pantothenic acid-deficient + A.C.E.[‡]
1:2560	1:1280	1:80	1:20
1:2560	1:2560	1:80	1:20
1:1280	1:1280	1:80	1:80
	1:5120		0

[*] Titers for individual rats are presented.

[†] Inanition controls received 300 γ of pantothenic acid daily. Rats of Groups I and II were "paired-weighed" with those of Groups III and IV, respectively, throughout experiment.

[‡] A.C.E. = Aqueous adrenal cortical extract.

2. *Pantothenic acid therapy.* Seven weanling rats fed the pantothenic acid-deficient diet for 6 weeks were immunized and their serum antibody titers determined. As indicated in the 2nd column of Table IV detectable titers were observed in only 2 animals. Four days later the deficient rats were injected intraperitoneally with 10 mg of calcium pantothenate on 2 successive days. In addition, these animals received 300 γ of pantothenic acid daily *per os* for the remainder of the experiment. Blood samples for antibody determinations were taken periodically as indicated in Table IV. It is evident that the effect of pantothenic acid upon serum antibody levels is very gradual, becoming perceptible only after approximately 23 days of therapy. It is of interest that, although the antibody response to pantothenic acid was delayed, the growth stimulating effects of this vitamin were apparent immediately. Thus, the average daily weight gain during the 9 days following pantothenic acid injection was 7 g. Five normal controls maintained a constant antibody titer of 1:2560 throughout the investigation. An identical experiment employing 18 rats yielded the same results.

3. *Pyridoxine therapy.* In this study a procedure similar to the one just described

---

[§] Parke, Davis and Co.

8. Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1939, v54, 2247.

9. Daft, F. S., and Sebrell, W. H., *Ibid.*, 1940, v55, 1333.

10. Ashburn, L. L., *Ibid.*, 1940, v55, 1337.

11. Deanne, H. W., and McKibbin, J. M., *Endocrinology*, 1946, v38, 385.

12. Chase, J. H., White, A., and Dougherty, T. F., *J. Immunol.*, 1946, v52, 101.

TABLE IV. Effect of Pantothenic Acid Therapy Upon Antibody Titers of Rats Previously Immunized While in a Pantothenic Acid Deficiency State.*

Rat No.	Initial titer‡	Days on experiment†					
		1	9	23	37	51	65
1	0	0	0	1:40	1:160	1:320	1:320
2	0	0	0	1:40	1:320	1:640	1:640
3	0	0	0	1:40	1:640	1:640	1:640
4	1:40	0	0	0	1:40	1:160	1:160
5	0	0	1:20	1:20	1:1280	1:2560	1:2560
6	0	0	0	1:40	1:640	1:640	1:640
7	1:80	1:40	1:40	1:80	1:1280	1:2560	1:2560

* Individual serum antibody titers are presented.
† Days after the second vitamin injection.
‡ Initial titers were determined 4 days prior to pantothenic acid administration.

TABLE V. Effect of Pyridoxine Therapy Upon Antibody Titers of Rats Previously Immunized While in a Pyridoxine Deficiency State.*

Rat No.	Initial titer‡	Days on experiment†					
		3	11	32	46	60	82
1	0	0	0	1:160	1:160	1:640	1:640
2	1:10	1:20	1:20	1:160	1:320	1:640	1:640
3	1:40	1:40	1:80	1:640	1:640	1:640	1:640
4	1:40	1:40	1:160	1:1280	1:1280	1:2560	1:2560
5	1:80	1:80	1:160	1:640	1:640	1:1280	1:2560
6	1:20	1:80	1:80	1:1280	1:2560	1:2560	1:2560
7	1:80	1:80	1:160	1:320	1:640	1:640	1:1280

* Individual serum antibody titers are presented.
† Days after the pyridoxine injection.
‡ Initial titers were determined 2 days prior to pyridoxine administration.

was employed. Seven weanling rats fed the pyridoxine-deficient diet for 5 weeks were immunized and their antibody titers ascertained (column II, Table V). Two days later these deficient animals were injected intraperitoneally with 5 mg of pyridoxine and for the remainder of the experiment they received 50 γ of the vitamin daily *per os*. Periodic antibody determinations revealed (Table V) that the administration of pyridoxine resulted in a gradual elevation of antibody levels, the effect becoming pronounced in most animals only on the 32nd day. This response to pyridoxine was, therefore, comparable to that obtained with pantothenic acid therapy (Table IV). Pyridoxine administration also resulted in a marked growth stimulation in all deficient rats as evidenced by an average daily weight gain of 7 g during the first 11 days following the vitamin injection. Five normal control animals maintained a constant antibody titer of 1:2560 throughout the experiment.

Discussion. The data presented in this paper demonstrate that rats immunized while receiving a complete diet were able to maintain high serum antibody titers during a protracted period in which either pantothenic acid or pyridoxine was removed from the diet. This finding suggests that neither a pantothenic acid nor a pyridoxine deficiency results in an accelerated rate of antibody destruction. In view of the fact that pronounced deficiencies of these vitamins were produced during the experimental period, it does not seem likely that an accelerated rate of antibody destruction would be counterbalanced by a corresponding increase in the rate of antibody synthesis. Further information on this point could be gained by the utilization of "tagged" antibodies to compare the "turnover" rate of serum antibodies in normal and vitamin-deficient rats(13,14).

The previous observations that a suppression of circulating antibodies occurs in panto-

---

13. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, v144, 545.

thenic acid(1,5) or pyridoxine-deficient(1,2) rats have been further verified in the present experiments. The lag in the increase of serum antibody titers following vitamin therapy in such rats appears to indicate that neither pantothenic acid nor pyridoxine is concerned with the release of preformed antibodies. In this connection Daft *et al.*(9) and Ashburn(10) have presented histological evidence that adrenal hemorrhage, atrophy, and necrosis in pantothenic acid-deficient rats can be repaired in 6 to 14 days by the daily administration of 100 γ of pantothenic acid. In the present experiments, although the pantothenic acid-deficient rats received 20 mg of pantothenic acid intraperitoneally and 300 γ of the vitamin daily *per os*, the serum antibody titers remained negligible until the 23rd day at which time they gradually began to increase reaching normal levels only after 51 days of vitamin therapy (Table IV). If pantothenic acid were concerned with a release or distribution mechanism functioning through the adrenal cortex a more immediate elevation of serum antibody titers to normal values would be expected. The gradual increase in serum antibody levels following pantothenic acid or pyridoxine therapy may be attributed to the corresponding slow rate of antibody synthesis catalyzed by the vitamin.

The evidence accumulated to date, albeit indirect in nature, suggests that both pantothenic acid and pyridoxine are concerned with the processes of antibody synthesis.

Summary. 1. Rats immunized while receiving a complete diet maintained high serum antibody titers during a protracted period of time in which either pantothenic acid or pyridoxine was removed from the diet. 2. Subcutaneous administration of aqueous adrenal cortical extract either immediately following each antigen injection or 3 weeks *after* immunization was ineffective in increasing the serum antibody titers of either pantothenic acid-deficient rats or their inanition controls. 3. Administration of massive doses of pantothenic acid or pyridoxine to rats immunized while in a corresponding vitamin deficiency state produced only a very gradual increase in serum antibody titers. Relatively normal levels were reached approximately two months after vitamin repletion. 4. These findings suggest that pantothenic acid and pyridoxine function in the processes of antibody synthesis.

---

14. Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R., and Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 301.

Received February 13, 1951. P.S.E.B.M., 1951, v76.

Circulating Antibodies in Vitamin Deficiency States. Pantothenic Acid-sparing Action of DL-Methionine. (18590)

PETER P. LUDOVICI, A. E. AXELROD,[†] AND BETTINA B. CARTER.
(Introduced by Ralph R. Mellon)

From the Institute of Pathology, The Western Pennsylvania Hospital, and the Department of Chemistry, University of Pittsburgh.

Previous experiments have shown that a dietary source of pantothenic acid is required for the production of circulating antibodies in the rat(1,2). Nelson and co-workers(3,4) have demonstrated the sparing action of high casein diets upon the pantothenic acid requirements of the rat for growth and survival. Recently, these authors(5) reported that DL-

---

[*] This investigation was supported by grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, the National Vitamin Foundation, Inc., and Hoffman-La Roche, Inc.

[†] Present address: Institute of Pathology, Western Reserve University, Cleveland, O.

1. Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 137.

methionine also possessed a marked pantothenic acid-sparing action. It, therefore, seemed of interest to determine whether DL-methionine could also spare the requirement of pantothenic acid for antibody production.

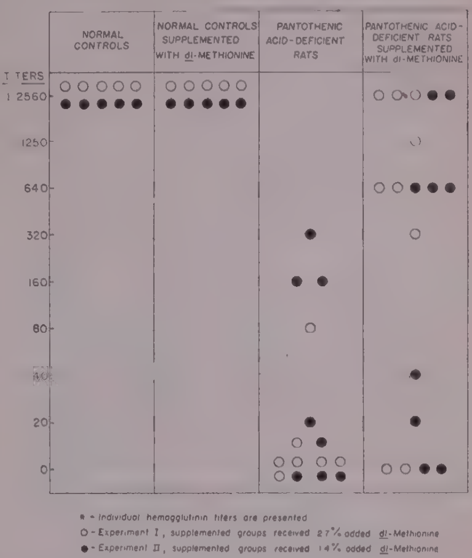
The effect of β-alanine upon antibody response in pantothenic acid-deficient rats has also been investigated.

Methods. The diets of the control and pantothenic acid-deficient animals were identical with those described in the preceding paper(6). The control diet (containing the complete vitamin supplement) furnished 300 γ of pantothenic acid and 50 γ of pyridoxine daily. DL-methionine was added to the diet at the expense of the sucrose, while the desired daily dosage of β-alanine was furnished in the vitamin pill. Male weanling albino rats were employed and, when indicated, their diets were supplemented with either DL-methionine or β-alanine from the onset of the experiment. The antigen, immunization procedure, and the determination of serum antibody titers were identical with those described in the preceding paper(6).

Experimental. A. *Pantothenic acid-sparing action of* DL-*methionine.* These studies were performed to determine the effect of DL-methionine upon the antibody response of pantothenic acid-deficient as well as normal rats. Two separate experiments, identical except for the DL-methionine content of the supplemented diets, were conducted. Levels of 2.7 and 1.4% DL-methionine were added to the diets of the DL-methionine supplemented groups of experiments I and II respectively. In each experiment the animals were distributed into 4 groups as indicated in Table I. All rats were fed their respective diets *ad libitum* throughout the investigation. The animals were immunized during the 7th week on experiment.

TABLE I

The Effect of dl-Methionine upon Serum Antibody Titers*



The individual hemagglutinin titers presented in Table I reveal that none of the unsupplemented pantothenic acid-deficient animals possessed serum antibody titers higher than 1:320. In contrast 60% of the pantothenic acid-deficient rats supplemented with DL-methionine exhibited titers of 1:640 or higher. It is also evident that additional DL-methionine had no effect upon the antibody titers of normal controls.

Supplementation with DL-methionine had no influence upon (1) the growth of either pantothenic acid-deficient rats or normal controls and (2) the severity of the pantothenic acid deficiency symptoms. Five of the 20 pantothenic acid-deficient rats and 2 of a comparable DL-methionine-supplemented group succumbed prior to immunization. Thus, the improved growth and survival observed by Nelson and Evans(5) were not demonstrable in our experiments. It should be noted, however, that Nelson and co-workers (3,4) reported that the pantothenic acid sparing effect of a high casein diet became more pronounced as the experimental period was extended beyond 60 days. Similar data on the time required for the pantothenic acid-

---

2. Ludovici, P. P., Axelrod, A. E., and Carter, B. B., *Ibid.*, 1949, v72, 81.

3. Nelson, M. M., and Evans, H. M., *Ibid.*, 1945, v60, 319.

4. Nelson, M. M., Nouhuys, F. V., and Evans, H. M., *J. Nutrition*, 1947, v34, 189.

5. Nelson, M. M., and Evans, H. M., *Amer. Chem. Soc.*, Abst. of Papers, 1949.

6. Ludovici, P. P., Axelrod, A. E., and Carter, B. B., in press.

TABLE II. Influence of β-alanine Upon Antibody Response and Growth of Pantothenic Acid-Deficient Rats.

Group	No. of rats	Body wt*		Antibody titers	
		Initial	Final†	Range	Avg
I. Normal control	5	42	223		1:2560‡
II. Pantothenic acid-deficient	10	44	89	1:5–1:320	1:148
III. Pantothenic acid-deficient + β-alanine	10	45	94	1:5–1:320	1:143

* Group avg in g.

† At the time of bleeding.

‡ All 5 rats had a titer of 1:2560.

sparing action of DL-methionine to become apparent have not been presented by these authors(5). Our failure to observe a significant improvement in growth and survival may be attributed to the shorter experimental period employed in the present experiments.

B. *Effect of β-alanine upon antibody response of pantothenic acid-deficient rats.* This experiment was conducted to determine whether β-alanine could substitute for pantothenic acid in the processes of antibody production. Twenty-five weanling rats were distributed into 3 groups as indicated in Table II. Each animal of Group III received 0.5 mg of β-alanine daily. The experimental diets were fed *ad libitum* to all rats and immunization was instituted during the 5th week.

It is evident from the results summarized in Table II that β-alanine cannot replace pantothenic acid for either antibody production or growth in the rat. The latter observation confirms the opinion of El-Sadr *et al.*(7) that the slight growth stimulation observed by Hoffer and Reichstein(8) was not significant.

Summary. 1. In albino rats DL-methionine possesses a significant sparing action upon the pantothenic acid requirement for antibody production. 2. β-alanine cannot replace pantothenic acid in the processes of antibody synthesis.

---

7. El-Sadr, M. M., Hind, H. G., Macrae, T. F., Work, C. E., Lythgoe, B., and Todd, A. R., *Nature,* 1939, v144, 73.
8. Hoffer, M., and Reichstein, T., *Ibid.,* 1939, v144, 72.

Received February 13, 1951. P.S.E.B.M., 1950, v76.

Studies on β-Glucuronidase from *E. coli.* (18591)

HENRY J. BUEHLER,* PHILIP A. KATZMAN, AND EDWARD A. DOISY.

From the Department of Biochemistry, Saint Louis University School of Medicine, Saint Louis, Mo.

Although the preparation of β-glucuronidase from mammalian tissues has been studied by Masamune(1), Oshima(2), Fishman(3-6) and others(7-11), little attention has been devoted to microorganisms as a source of this important enzyme. That bacteria may possess β-glucuronidase activity has been suggested by the reports of Cohen and Marrian (12) and Patterson (13) that the estrogen of pregnancy urine was liberated by putrefaction. Barker, Brooksbank, and Haslewood (14) found that pregnanediol glucuronide and in some experiments glucuronic acid which was presumably liberated from the conjugate, were destroyed by some strains of *Staphylococcus albus*. Earlier, Bucher and Geschickter (15) had presented evidence which indicated that the liberation of pregnanediol in urine from pregnanediol glucuronide was brought about by the action of a bacterial enzyme. Our interest in β-glucuronidase lay in its use as a means of hydrolyzing steroid glucuronides. In an earlier paper, we reported the production of this enzyme (16) by *E. coli* and the hydrolysis of urinary estrogen and 17-ketosteroid conjugates by the bacterial culture fluid (17). The preparation of potent solutions of β-glucuronidase by the method described in this paper has been carried out by 3 other workers in this laboratory and one (18) of them has reported on the hydrolysis of conjugates of reducing lipids of urine by such solutions.

---

* Atomic Energy Commission Pre-doctoral Fellow in the Biological Sciences.

1. Masamune, H., *J. Biochem. Japan,* 1934, v19, 353.
2. Oshima, G., *J. Biochem. Japan,* 1936, v23, 305.
3. Fishman, W. H., *J. Biol. Chem.,* 1939, v127, 367.
4. Fishman, W. H., *J. Biol. Chem.,* 1940, v136, 229.
5. Fishman, W. H., *J. Biol. Chem.,* 1947, v169, 7.
6. Bernfeld, P., and Fishman, W. H., *Arch. Biochem.,* 1950, v27, 475.
7. Graham, A. F., *Biochem. J.,* 1946, v40, 603.
8. Mills, G. T., *Biochem. J.,* 1948, v43, 125.
9. Sarkar, N. K. and Sumner, J. B., *Arch. Biochem.,* 1950, v27, 453.
10. Talbot, N. B., Ryan, J., and Wolfe, J. K., *J. Biol. Chem.,* 1943, v151, 607.
11. Levy, G. A., *Nature,* 1947, v160, 54.

This report describes the more important points which must be taken into account in preparing potent solutions of β-glucuronidase from cultures of *E. coli*.

Experimental. The basal medium employed in our survey of bacteria as a source of β-glucuronidase consisted of 1.0% Difco bacto-peptone, 0.3% Difco bacto-beef extract, 0.5% sodium chloride, and 3.0% sodium glycerol phosphate. β-Glucuronidase activity was determined by the hydrolysis of sodium phenolphthalein glucuronidate, a chromogenic substrate introduced by Talalay, Fishman, and Huggins (19). Of twenty-three bacteria tested, only *C. xerose, C. hoffmani,* and *E. coli* demonstrated β-glucuronidase activity. *E. coli* was selected for further study for the production of the enzyme, because of the ease with which this organism can be cultured. However, not all strains of *E. coli* possess the capacity to produce β-glucuronidase. The strain (available on request) employed in our studies was isolated from a case of cystitis.

Culture pH. The optimum pH for the production of β-glucuronidase was determined by growing *E. coli* in the basal medium at various pH levels for 5 days at 25°C.† Using the Beckman pH meter, the pH of the medium was adjusted by the addition of HCl or NaOH. Autoclaving and subsequent incubation did not alter the original pH of the medium. After incubation, the cultures were centrifuged for 1 hour at 3000 r.p.m. (International centrifuge, Size 1) to remove the bacterial debris, and the clear culture fluids were adjusted to pH 7.0‡ and analyzed for their β-glucuronidase activity. Aliquots of the various culture fluids were incubated with sodium phenolphthalein glucuronidate at 37°C for 24 hours. Chloroform, which does not inhibit bacterial β-glucuronidase, was added as a preservative during the incubation. The optimum pH for β-glucuronidase production was found to be approximately 7.3. At pH 6.5 and 8.0, the enzyme production was respectively 26% and 38% less than at pH 7.3.

Stimulation by substrate. Because of the well known influence of substrate upon the

---

12. Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1934, v28, 1603.

13. Patterson, J., *Brit. Med. J.*, 1937, v2, 522.

14. Barker, M., Brooksbank, B. W. L., and Haslewood, G. A. D., *Nature*, 1948, v162, 701.

15. Bucher, N. L. A., and Geschickter, C. F., *Endocrinology*, 1940, v27, 727.

16. Buehler, H. J., Katzman, P. A., and Doisy, E. A., *Fed. Proc.*, 1949, v8, 189.

17. Buehler, H. J., Katzman, P. A., Doisy, P. P., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 297.

18. Kinsella, Jr., R. A., Doisy, R. J., and Glick, Jr., J. H., *Fed. Proc.*, 1950, v9, 190.

19. Talalay, P., Fishman, W. H., and Huggins, C., *J. Biol. Chem.*, 1946, v166, 757.

† This strain of *E. coli* produced much less β-glucuronidase when grown at 37°C.

‡ In the course of developing the procedure for potent preparations of β-glucuronidase, many assays were conducted before the pH of optimum activity was established. Some were carried out at pH 7; some at pH 7.3, but in each case the pH was kept constant for the series of each experiment.

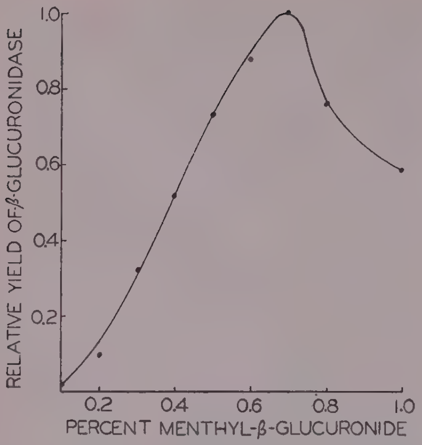


FIG. 1.

Influence of concentration of menthyl-β-glucuronide in the culture medium on production of β-glucuronidase by *E. coli.*

production of enzymes, a β-glucuronide was added to the growing cultures of *E. coli* to ascertain whether this organism would respond by producing larger amounts of β-glucuronidase. Varying amounts of menthyl-β-glucuronide, prepared according to Williams (20), were added to the basal medium at pH 7.3 and after inoculation the cultures were incubated for 5 days at 25°C. Since we had found that shaking the growing cultures increases the yield of the enzyme (see below), these cultures were kept on a shaking machine, operating at 75-100 r.p.m., during the incubation period. After filtration of the culture through paper to remove the liberated menthol and centrifugation to remove bacterial debris, the clear culture fluids were assayed for β-glucuronidase activity. It was found that the greatest yield of the enzyme (Fig. 1) was produced in a medium containing 0.7% of menthyl-β-glucuronide. The factors responsible for the apparent fall of activity in media containing higher concentrations of menthyl-β-glucuronide have not been studied.

Influence of surface area. The influence of the surface area on the production of β-glucuronidase was determined by incubating equal volumes of culture containing 0.7% of menthyl-β-glucuronide at pH 7.3 in flasks of different capacities. Thus the surface area per unit volume of culture varied with the size of the flask employed. These were cultured without shaking for 5 days, after which time assays were made for enzymatic activity. It was found that the production of enzyme was increased with increasing size of the flask in which the culture was grown. This experiment was repeated with the same series of flasks but employing constant agitation during the incubation period. A shaking machine operating at 75-100 r.p.m. was used for this purpose. This treatment completely overcame the effect of surface area, since all the cultures produced the same amount of β-glucuronidase, irrespective of the capacity of the flasks in which they were grown (Fig. 2). From these studies, it seems likely that an adequate amount of oxygen is necessary for the optimum production of β-glucuronidase by *E. coli.*

Incubation period. To determine the optimum period of incubation for the production of β-glucuronidase, cultures at pH 7.3 containing 0.7% of menthyl-β-glucuronide were grown with continuous shaking for varying lengths of time ranging from 2 to 11 days.

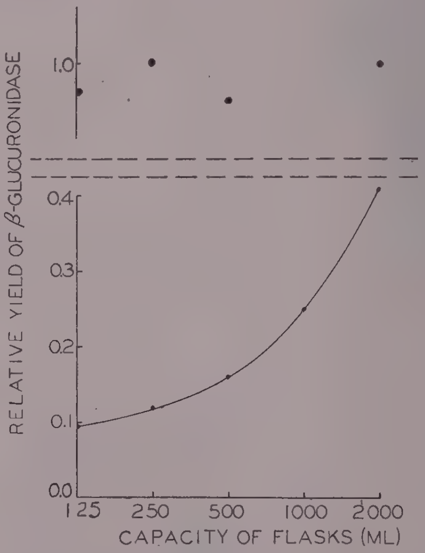


FIG. 2.

Influence of surface area of culture on production of β-glucuronidase by *E. coli.* Curve shows results when flasks were stationary, while effect of shaking them is indicated by points above broken lines.

---

20. Williams, R. T., *Biochem. J.*, 1938, v32, 1849.

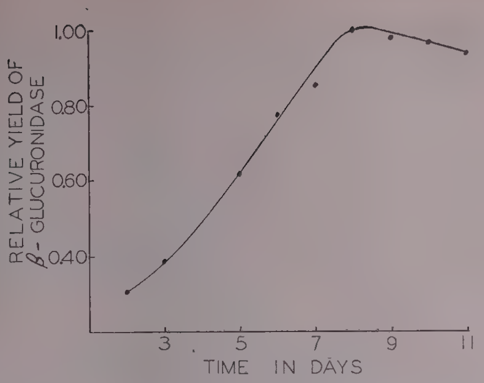


FIG. 3.

Effect of length of incubation period on production of β-glucuronidase by *E. coli.*

It was found that an incubation period of eight days gave the greatest amount of enzyme (Fig. 3).

Method of preparation. As a result of the foregoing studies, we have adopted the following procedure for the preparation of bacterial β-glucuronidase. Fifty ml of medium containing 1.0% Difco bacto-peptone, 0.3% Difco bacto-beef extract, 0.5% sodium chloride, 3.0% sodium glycerol phosphate (Mallinckrodt, U.S.P.), and 0.7% menthyl-β-glucuronide at pH 7.3 are placed in a 250 ml Erlenmeyer flask and autoclaved for 15 minutes at 15 pounds pressure. After cooling, the medium is inoculated with *E. coli* which had been carried on nutrient agar slants. The inoculated medium is incubated for 8-10 days at 25°C with continuous shaking by a rotary shaking machine operating at 75-100 r.p.m. The pH of the culture remains at 7.3 during the incubation period. The culture is then filtered through paper to remove the suspended menthol, and the filtrate is centrifuged in the cold room at 3500 r.p.m. to separate the bacterial cells from the enzyme solution. The resulting clear culture fluid without further purification can be used as the β-glucuronidase preparation. Such preparations have been stored in the refrigerator at 5°C for months without any detectable loss of activity. Stable powders can also be prepared by lyophilization. The culture fluid is rich in phosphatase activity as demonstrated by the hydrolysis of phenolphthalein phosphate. However, phenolic or alcoholic sulfatase could not be detected. Other enzymic activity was not studied.

Method of assay. The determination of the glucuronidase activity of our potent enzyme preparations is carried out by a modification of the Talalay, Fishman and Huggins procedure(19). The culture fluid is adjusted to pH 6.2 with HCl after it has been filtered and centrifuged. One ml is diluted to 10 ml with distilled H_2O; to 2 ml of this is added 1 ml of a sodium phenolphthalein glucuronidate solution containing the equivalent of 450 μg of phenolphthalein per ml, and the mixture is incubated for 4 hours at 37°C. Then to 1 ml of this incubated mixture are added 5 ml of 0.4 molar glycine buffer (pH 10.4) and the color is read in a colorimeter using a green filter (range 500-570 mμ) to determine the micrograms of phenolphthalein liberated.

$$\frac{\gamma\text{-Phenolphthalein x } 3 \text{ x } 10}{2} = \text{micrograms of}$$

phenolphthalein liberated per ml of original culture fluid. One ml of enzyme preparations made and assayed according to the above procedures usually hydrolyzed phenolphthalein glucuronide equivalent to 1500-2500 μg of phenolphthalein.

Effect of pH on β-glucuronidase. To study the effect of pH on bacterial β-glucuronidase activity, thoroughly dialyzed preparations of the enzyme were incubated with sodium phenolphthalein glucuronidate and with sodium estriol glucuronidate.§ The incubation mixtures were buffered with 0.067 molar phosphate at pH levels ranging from 4.5 to 8.5. The optimum pH for the hydrolysis of sodium phenolphthalein glucuronidate was approximately 6.2. However, with sodium estriol glucuronidate no variation in rate of hydrolysis was found between pH 4.5 and 7.0, but above pH 7.0 the activity fell off very abruptly. Because the optimum pH range was so broad in this case, duplicate determinations were made with 3 different preparations of β-glucuronidase. The results of these experiments which are summarized in Fig. 4, were identical.

---

§ Our thanks are due to Professor G. F. Marrian for supplying estriol monoglucuronide.

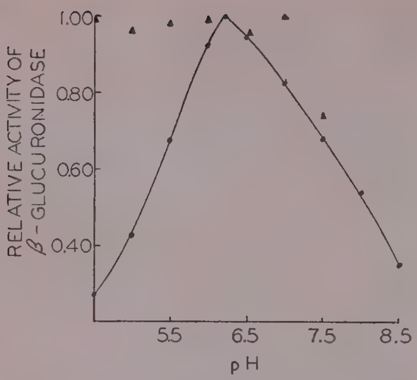


Fig. 4.

Influence of pH on activity of β-glucuronidase produced by *E. coli.*

●—Phenolphthalein-mono-β-glucuronide as substrate.

▲—Estriol-β-glucuronide as substrate.

Some properties of the β-glucuronidase. As the main object of this investigation was to prepare adequate quantities of β-glucuronidase for use in the determination of steroid glucuronides, properties of the enzyme have not been extensively studied. Dialysis of the culture fluid containing the enzyme caused the loss of at least one-half of the activity. This loss of activity was not prevented by controlling the temperature and the pH during dialysis. This bacterial β-glucuronidase is readily inhibited by ammonium chloride, a concentration of 0.1% of this salt causing a 20% reduction of activity. For this reason, the free acid and not the ammonium salt of menthyl-β-glucuronide is used in the culture medium for the preparation of the enzyme. Sodium chloride, in concentrations above 1.0%, decreases the activity of the enzyme appreciably.

Summary. Potent culture fluid preparations of β-glucuronidase are obtained when *E. coli* is grown at 25°C and pH 7.3 in a simple medium containing menthyl-β-glucuronide. The optimum incubation time is 8 days when continuous agitation is employed. The optimum pH for the hydrolysis of phenolphthalein mono-β-glucuronide is 6.2, while that for estriol-β-glucuronide is 4.5-7.0. Bacterial β-glucuronidase is readily inhibited by ammonium ion. Dialysis markedly decreases the enzymatic activity of our preparations. The culture fluid contains phosphatase but no sulfatase activity.

Received February 14, 1951. P.S.E.B.M., 1951, v76.

Biological Activity of Alkali Treated Aureomycin. (18592)

A. C. Dornbush, J. J. Oleson, A. L. Whitehill, and B. L. Hutchings.
From the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Recently, the growth promoting effect of aureomycin on the chick(1,2), the turkey(3), the rat(4,5), and the pig(6) have been reported. The general interpretation of these results has stressed the probable effect of the antibiotic on the intestinal flora of the test animal. The inactivity of intramuscularly administered aureomycin or penicillin(1,7) was in accord with this hypothesis.

Data presented by Stokstad and Jukes(2) suggested that aureomycin after microbiological inactivation with alkali was still active for the chick. As these data were not in agreement with the results we had obtained, it was decided to investigate more fully the biological

---

1. Whitehill, A. R., Oleson, J. J., and Hutchings, B. L., Proc. Soc. Exp. Biol. and Med., 1950, v74, 11.

2. Stokstad, E. L. R. and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1950, v73, 523.

3. Stokstad, E. L. R. and Jukes, T. H., *Poultry Science,* 1950, v29, 611.

4. Oleson, J. J., Hutchings, B. L., and Whitehill, A. R., *Poultry Science,* 1950, v29, 774.

5. Stern, J. R., and McGinnis, J., *Proc. A.C.S. Meetings,* Sept. 1950, p33C.

6. Carpenter, L. E., *Arch. Biochem.,* 1950, v27, 469.

7. McGinnis, J., Berg, L. R., Stern, J. R., Wilcox, R. A. and Bearse, G. E., *Poultry Science,* 1950, v29, 771.

TABLE I. Antibacterial Spectrum of Aureomycin, Aureomycin Mash, and Alkali Treated Aureomycin Mash Samples.

	Minimal concentrations* suppressing visible growth after 24 hr incubation				
			Alkali treated mash		
Organism	Aureomycin, γ/ml	Aureomycin mash, γ/ml	pH 13.0, γ/ml	1 cc of 5N NaOH, γ/ml	5 cc of 5N NaOH, γ/ml
Shigella gallinarum	1.56	1.56	1.56	1.56	1.56
Bacillus subtilis	.048	.048	.048	.048	.048
Staphylococcus albus	.195	.195	.195	.195	.195
Staphylococcus aureus	.097	.097	.097	.097	.097
Bacillus cereus	.048	.048	.097	.048	.024
Escherichia coli	1.56	1.56	1.56	1.56	1.56

* The solutions were diluted to equivalent concentrations based on residual antibiotic activity as determined by assay with *Staphylococcus aureus.*

activity of alkali inactivated aureomycin and to study the stability of aureomycin in pure solution and in the presence of certain additives.

Experimental. The paper of Stokstad and Jukes mentioned heating whole aureomycin mash with an excess of 0.5N sodium hydroxide followed by neutralization and concentration. Experiments were carried out as follows: Whole aureomycin mash from an industrial fermentation was divided into four 50 ml portions. Three of the portions were adjusted to pH 13 by the addition of 5N NaOH. One ml and 5 ml of 5N NaOH were added respectively to 2 of the flasks. The volume in each flask was then adjusted to 60 ml. The 3 flasks with alkali were heated with flowing steam in an autoclave for 30 minutes. At the end of this time each sample was extracted with 9 volumes of acid aqueous acetone (1 part 4N HCl, 13 parts acetone and 6 parts water) and the extracts were assayed turbidimetrically for aureomycin activity(8). When compared with the unheated aureomycin mash, the sample which had been adjusted to pH 13 and then heated retained 70% of its activity. The sample receiving 1 ml of 5N NaOH after being adjusted to pH 13 was 52% as active as the control whereas the sample receiving 5 ml of 5N NaOH was 41% as active. From the data it is apparent that even after heating for rather extended periods in alkali, appreciable antibiotic activity remains in the "inactivated" mash. In contrast to this, solutions of pure aureomycin are rapidly inactivated by these procedures.

That the activity remaining is due to unchanged aureomycin is not answered by the above data. To gain further information on this point, the anti-bacterial spectra and the behavior of the alkali treated material on paper chromatography was investigated. The spectra of the extracts of the various alkali treated samples were identical to that of aureomycin (Table I). The diluted mash extracts were applied to 0.5 inch paper strips and developed for 17 hours at room temperature in an atmosphere saturated with N HCl—n butanol (1:1 by volume). The R_f value for the residual antibiotic in all samples was identical with the value of 0.75 obtained by Arnow and Emerick(9) for aureomycin.

The protective effect of the whole mash on aureomycin with respect to inactivation by alkali suggested that perhaps the presence of colloidal materials would exert a similar protective action. In order to test this theory various colloidal substances were suspended in water, a solution of aureomycin hydrochloride was added and the pH of each was adjusted to 9.0 by the addition of 5N sodium hydroxide. The suspensions were placed in the 37° incubator and samples were removed for microbiological assay daily. The results are shown in Table II. It is apparent that the addition of any of a number of colloids or solutions that contain colloidal or suspended

---

8. Dornbush, A. C. and Pelcak, E. J., *Ann. N. Y. Acad. Sci.*, 1948, v51, 218.

9. Arnow, P. and Emerick, M., *Science*, in press.

TABLE II. Protective Action of Various Colloids or Suspended Materials Upon Aureomycin in Aqueous Solution at pH 9.0 and 37°C.

Substance added	Conc., %	Aureomycin conc., %	% destruction at, hr 24	48	72	96
0		.1	99.2	99.5	99.8	99.9
India gum	1.0	.1	22	37	41	47
Gum arabic	5.0	.1	3	18	24	36
Gum tragacanth	1.5	.1	27	25	39	41
Egg albumin	5.0	.1	17	22	21	37
Tomato juice	90.0	.025	50	69	78	84

TABLE III. Growth Promoting Activity of Alkali Inactivated Aureomycin on the Chick.

	Avg wt and numbers alive ()		
Supplement per kg diet	Initial, g	15 days, g	28 days, g
1. Basal (1) + 90 γ B_{12}/kg	41(9)	148(9)	347(9)
2. As 1 + 10 mg aureomycin	40(9)	171(9)	369(9)
3. '' + 25 mg ''	40(9)	171(9)	385(8)*
4. '' + 10 mg alkali treated aureomycin	40(9)	147(7)	338(7)
5. '' + 25 '' '' '' ''	38(9)	145(8)	305(8)
6. '' + 50 '' '' '' ''	44(9)	150(9)	319(9)
7. '' + 100 '' '' '' ''	43(9)	168(9)	354(9)

* Significant at 5% level.

materials markedly stabilizes solutions of aureomycin to the destructive effects of alkali.

The results so far presented indicate that treatment of the whole aureomycin mash by alkali under the present conditions does not cause complete inactivation of the antibiotic. The activity reported in the experiments of Stokstad and Jukes could thus be attributed to unchanged aureomycin.

The possibility still existed that the microbiological activity of the mash which was due to residual aureomycin, did not truly represent the activity for the chick and that the alkali treated aureomycin would be active in the latter test. To test this hypothesis the product arising from the treatment of aureomycin with dilute alkali was isolated and tested on the chick. In order to avoid any drastic degradation of the aureomycin molecule, the alkali treatment was carried out essentially as described by Kelsey and Goldman(10). Ten grams of aureomycin hydrochloride was dissolved in 25 ml of water with just enough 10% NaOH to effect complete solution. Two hundred and fifty ml of phosphate buffer (pH 10.5, 2N) was then added. The solution was heated to 90-95° for 5 minutes and then refluxed for 5 minutes (24 hours at 25° effects the same transformation). The mixture was cooled, adjusted to pH 5.4 with hydrochloric acid and the precipitate collected on a filter. The compound was dissolved in dilute alkali, decolorized with 5 grams of Norite A and the solution then adjusted to pH 5.4. The crystalline compound was collected and dried. A small sample was recrystallized from dilute hydrochloric acid. Analyses. C, 50.67; H, 4.90; N, 5.35; Cl, 13.81.

When tested against *Staphylococcus aureus*, the compound exhibited less than 0.04% of the antibiotic activity of aureomycin. The effect of the compound on the growth of the chick was determined as previously described (1). These results are summarized in Table III. The compound is inactive for the chick at the various levels tested.

Discussion. The foregoing data suggest that rather drastic conditions would be necessary to completely inactivate aureomycin in the whole fermentation mash. The residual aureomycin is most probably the active agent in the alkali "inactivated" product of Stokstad and Jukes rather than an antibacterially inert product that retains activity for the

---

10. Kelsey, H. A. and Goldman, L., *J. Clin. Invest.*, 1949, v28, 1048.

chick. This view is strengthened by the fact that a compound which is formed from aureomycin by treatment with dilute alkali is inactive for the chick and exhibits only a fraction of the antibacterial activity of aureomycin. These data further support the view that the antibiotic activity of a compound and its growth promoting activity for animals are related. The specific activity of each compound as regards its growth promoting effect is most probably correlated with the antibiotic spectrum of the compound and with the availability and stability of the substance in the intestinal tract of the animal. These factors could account in part for the fact that certain antibiotics are superior to others as growth stimulators.

Several substances when added to solutions of aureomycin stabilize the latter against inactivation by alkali. The exact mode of this action is unknown.

Summary. Alkali treated *Streptomyces aureofaciens* fermentation liquor contained considerable unchanged aureomycin. The pure antibiotic is inactivated by similar treatment. The product arising from mild alkaline inactivation of aureomycin was isolated and found to have essentially no antibacterial activity and no growth stimulating activity for the chick.

We are indebted to Dr. J. H. Williams, Director of Research, for his constant interest and advice. It is a pleasure to express our appreciation to Mr. L. M. Brancone and staff for the microanalyses, to Mr. Carl Wolf and Mrs. Angela Goldman for the preparation of the alkali inactivated aureomycin and to Mr. Peter Arnow for the chromatography of the various solutions.

Received February 15, 1951. P.S.E.B.M., 1951, v76.

Pathological Changes in Adrenal Cortex of Mouse During Experimental Meningococcal Infection.* (18593)

DAVID RUML† AND MARJORIE BOHNHOFF. (Introduced by C. Phillip Miller)
From the Department of Medicine, University of Chicago.

Importance has been attached to hemorrhage(1,2) and other changes in the adrenal cortex, particularly the "tubular degeneration" of Rich(3), in patients dying from fulminating meningococcal sepsis. These reports suggested that similar changes might be reproduced in animals experimentally infected with meningococcus.

The following observations are limited to microscopic changes in the adrenal cortex such as changes in the amount and distribution of fat, hyperemia and inflammatory infiltration.

Methods and materials. The meningococcus employed was a type I strain originally isolated from the blood stream of a child acutely ill with epidemic meningitis. A 6-hour culture of the organism was suspended in 4% mucin‡ and mice were inoculated intraperitoneally with 1.0 ml containing from 10^2 to 10^5 organisms. The virulence of this strain had been increased by mouse passage so that < 10 meningococci in mucin produced sepsis which was fatal within 48 hours. Animals which were inoculated with 10^2 organisms and remained untreated were moribund at 24 hours and rarely lived for 48 hours. No

---

* This investigation was supported, in part, by the U. S. Navy, Office of Naval Research, and, in part, by the A. B. Kuppenheimer Foundation of the University of Chicago.

† Present address of Lt. Ruml—Air Surgeon's Office, 14th Air Force, Robbins Field, Macon, Ga.

1. Moritz, A. R. and Zamcheck, N., *Arch. Path.*, 1946, v42, 459.

2. Daniels, W. B., *Arch. Int. Med.*, 1948, v81, 145.

3. Rich, A. R., *Bull. Johns Hopkins Hosp.*, 1949, v74, 1.

‡ Granular mucin, type 1701-W, supplied by the Wilson Laboratories, Chicago, Ill. The method of preparation is described by Miller and Bohnhoff(4).

4. Miller, C. P. and Bohnhoff, M., *J. Inf. Dis.*, 1948, v83, 256.

comparison therefore could be made between penicillin-treated and untreated mice after 24 hours. Mice injected with 10^5 meningococci died within 20 hours unless they were treated not later than 6 hours post-infection. Treatment consisted of a single dose of 200 units of penicillin§ administered subcutaneously in 0.5 ml of saline 6 hours after infection. The heart's blood of untreated mice was cultured on a meat digest agar(5) and meningococci were recovered in pure culture after 24 hours' incubation under reduced oxygen tension. The strains of mice¶ used were equally susceptible to the strain of meningococcus employed. The mice were adult males, 6 to 8 weeks of age, weighing 20 to 24 g.

Autopsy, staining and fixing.‖ In order to avoid postmortem changes, animals were killed at various times after infection and the kidneys removed with the untouched adrenal glands attached. The combined specimen was fixed for 7 days in a modified Bouin's solution.** The adrenal gland, attached to the superior pole of the kidney, was frozen and sectioned on a Spencer freezing microtome. The technic gave sections through the same regions of each gland and avoided changes which might be produced by manipulation. This was important because the adrenal gland is extremely delicate, and the slightest pressure will cause artifacts to appear in the microscopic sections(6,7). Of the 350 pairs of adrenals studied, 130 were stained with hematoxylin and eosin-azure and 220 were stained for fat with Sudan III (saturated solution in equal parts of 50% alcohol and Carbitol††) and counter-stained with hematoxylin. Both staining methods were satisfactory for the study of cellular changes, but only the fat stain was adequate for distinguishing lipid vacuoles. For this reason, most of the sections were stained for fat.

Anatomy of the mouse adrenal cortex. The adrenal cortex of the mouse is divided into 3 zones (See Fig. 1): (a) the zona glomerulosa, rounded groups of cells averaging 8 to 12 cells in depth. This zone normally contains relatively less fat than the second zone. (b) the zona fasciculata, the thickest zone which consists of radial columns of cells, and contains most of the fat principally in its outer two-thirds. (c) the zona reticularis ("embryonic, boundary zone, fetal or x-zone"), the structure of which depends upon the age and sex of the animals(7-10).

Controls. Adrenals were prepared from mice injected with mucin, penicillin and saline as well as from normal healthy controls and from animals which had been deprived of food and water.

Microscopic study. Each adrenal cortex was examined for the following details: 1. Cellular changes: such as cellular degeneration, zonal degeneration of the type described by Humphreys and Donaldson(11), "tubular degeneration"(3) and increased mitoses which are frequently seen in the pathological adrenal cortex(9,12,13). Changes in thickness of the cortex described as occurring "under stress"(14,15) were observed but could not be evaluated with sufficient ac-

---

§ The penicillin was supplied by the Abbott Laboratories, Lederle Laboratories, Eli Lilly and Co., and Schenley Laboratories,.

5. Miller, C. P. and Bohnhoff, M., *J. Inf. Dis.*, 1947, v81, 147.

¶ Maple Grove Rabbitry, Springfield, Mo. and CF No. 1 strain, Carworth Farms, New City, N. Y.

‖ This method of obtaining, fixing and staining was devised with the assistance of Mr. John H. Daniels of the Department of Medicine, University of Chicago.

** Fixative: 2 parts of glacial acetic acid; 75 parts saturated solution of picric acid; 25 parts of formaldehyde solution.

6. Hoerr, N., *Am. J. Anat.*, 1931, v48, 1.

7. Grollman, A., "The Adrenals," Williams and Wilkins, Baltimore, 1936.

†† Carbitol: Doethylene glycol monoethyl ether, a product of The Carbide and Carbon Corp.

8. Howard-Miller, E., *Am. J. Anat.*, 1927, v40, 251.

9. Deanesly, R., *Am. J. Anat.*, 1931, v47, 475.

10. Howard, E., *Am. J. Anat.*, 1938, v62, 351.

11. Humphreys, E. M. and Donaldson, L., *Am. J. Path.*, 1941, v17, 767.

12. Whitehead, R., *Br. J. Exp. Path.*, 1932, v13, 200.

13. Graham, G. S., *J. Med. Research*, 1916, v34, 241.

14. Sarason, E. L., *Arch. Int. Med.*, 1943, v71, 702.

15. Sayers, G., and Sayers, M. A., Recent Progress in Hormone Research, vII, Academy Press, Pincus, New York, 1948.

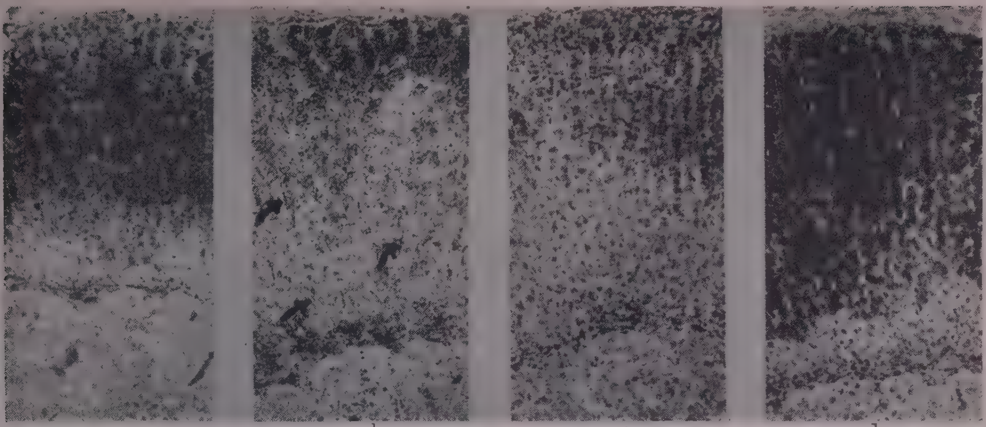


FIG. 1.

Sections of mouse adrenals showing depletion of fat in the zona fasciculata during meningococcal infection and its restoration after successful treatment with penicillin. All sections stained with Sudan III and counter-stained with hematoxylin eosin-azure. Mag. ×210.

(a) Normal control: Section shows, from above downward, the zona glomerulosa, the zona fasciculata, and the outer rim of the medulla.

(b) Untreated mouse: Killed 24 hr after infection with meningococcus. Blood culture positive. Adrenal cortex shows depletion of fat (compare with normal) and engorgement, hemorrhage, and inflammatory cell infiltration at cortico-medullary junction. Arrows indicate engorged capillaries.

(c) Penicillin treated mouse: Killed 24 hr after infection with meningococcus. Blood culture negative. Adrenal cortex shows more fat than untreated (b). Much of the light gray color in the inner half of the zona fasciculata represents fat in finely divided droplets. Less engorgement and inflammatory cell infiltration than (b).

(d) Penicillin treated mouse: Killed 48 hr after infection with meningococcus. Blood culture negative. Adrenal cortex shows a return to normal amount of fat.

curacy. 2. Areas of inflammatory cell infiltration. 3. Engorgement of capillaries and hemorrhage within the cortex and at the cortico-medullary junction. 4. Abnormalities in quantity and distribution of fat in the cortex. In estimating the amount of fat depletion and engorgement in adrenals from infected animals, allowance was made for normal variation as determined by examination of adrenals from control mice.

Results: The adrenal cortex of mice infected with meningococcus showed depletion of fat, engorgement and inflammatory cell infiltration. Most of the sections from infected mice showed generalized reduction of fat, most commonly seen in the zona glomerulosa and in the inner half of the zona fasciculata. Typical examples are shown in the microphotographs in Fig. 1 which includes a normal cortex for comparison. Moderate fat depletion occurred in untreated mice as early as one to 2 hours after infection and became conspicuous at 7 to 24 hours. Subsequent observations were possible only in the treated animals. Less than half of these mice at 28 to 48 hours showed depletion of fat, and only a few of them showed marked depletion, indicating that recovery was in progress.

In Table I the incidence of fat depletion and other changes is related to the course of the infection. It should be pointed out, however, that the degree of depletion, not recorded in the table, was most marked at those periods when it was found in the highest percentage of mice. It was of interest to note that in a few sections, the fat was concentrated in the zona glomerulosa and in a band in the zona fasciculata with or without a fat-free zone between the two. An occasional section showed fat only in the zona glomerulosa.

The adrenals of control mice receiving mucin, penicillin and saline showed no significant differences from the adrenals of normal stock animals. Engorgement was usually greatest at the cortico-medullary junction and areas of hemorrhage within the cortex

TABLE I. Changes in Adrenal Cortex of Mice Infected with Meningococcus.

Time after infection, hr	% of adrenals showing: Fat depletion		Engorgement		Inflammatory cell infiltration	
	UnR$_x$, %	R$_x$,* %	UnR$_x$, %	R$_x$,* %	UnR$_x$, %	R$_x$,* %
Uninfected controls	0	0	0	0	0	0
1–2	40	*	20	*	50	*
3–6	65	*	60	*	40	*
7–20	75	100	60	70	70	75
24	100	65	75	40	85	45
28–48	†	40	†	45	†	60

* 200 units of penicillin administered subcut. 6 hr after infection.

† No survivors among untreated mice.

were most frequently located in the zona fasciculata adjacent to the area of greatest cortico-medullary engorgement. Engorgement seldom extended more than half way into the zona fasciculata. Table I shows a comparison of the amount of engorgement in untreated mice and those treated with penicillin.

Inflammatory cell infiltration was greatest at or adjacent to the areas of greatest cortico-medullary engorgement, and the reaction seldom extended farther than the inner half of the zona fasciculata. (Table I). No other cellular changes: *e.g.*, mitoses, were observed in any of the sections.

Discussion. Fat depletion, engorgement and inflammatory cell infiltration occur in the adrenals of mice with experimental meningococcal sepsis. The extent of these changes was modified by treatment with penicillin which terminated the infection. Infected mice did not show increased mitosis, cellular degeneration or zonal degeneration which have been produced in animals by various infectious or toxic agents(3,9,11-13). They also lacked the tubular degeneration described by Rich in the adrenals of patients dying of fulminating meningococcal sepsis. It was not possible in these mouse adrenals to evaluate with sufficient degree of accuracy changes in thickness of the cortex such as had been reported in various inflammatory diseases (14).

Since the animals infected with meningococcus did not eat or drink, adrenals were examined in a small group of mice deprived of food and water for 48 hours. Fat depletion was not noted at 24 hours, but it was observed in a moderate degree at 48 hours. This degree of fat depletion at 48 hours was not so great as the depletion which occurred at 24 hours in infected untreated animals. No other abnormalities were seen in these adrenal glands.

Summary. Employing a technic which minimized the production of artifacts, a histological study was made of the adrenal glands of mice infected with meningococcus and also of appropriate controls. Depletion of fat, engorgement and inflammatory cell infiltration were demonstrated during the course of the infection. After successful treatment with penicillin the adrenal cortex showed a progressive recovery as manifested by reaccumulation of fat and reduction of hyperemia and inflammatory reaction.

Received February 16, 1951. P.S.E.B.M., v76, 1951.

Regeneration of Liver Tissue Following Partial Hepatectomy in Parabiotic Rats. (18594)

Alvin S. Wenneker and Noah Sussman. (Introduced by H. L. White)

From the Departments of Physiology and of Pathology, Washington University School of Medicine, St. Louis.

Liver regeneration in single albino rats has been observed as follows by Higgins(1). Rats were partially hepatectomized and killed by exsanguination at intervals up to 30 days postoperatively. The livers were weighed at autopsy and the percentage of regeneration calculated on the basis of liver to body weight ratios in a series of control rats. Franseen(2) employed a standardized procedure, removing 66.83% ± 3.54 of the liver by excising the median and left lateral lobes and leaving the posterior lobe. We confirmed Franseen's observations by performing such partial hepatectomies (lobectomy) on 5 rats, immediately killing them, weighing the remaining liver, and calculating the percentage of the liver removed. This turned out to be 67% ± 5, and the identical operation was then utilized in our experiments. We consider this to be a better measure of the percentage of liver removed than is given by comparing the weight of removed liver with the average of total liver weight of a control series of rats of comparable body weight.

The purpose of the present work was to observe liver regeneration in parabiotic rats as compared with a group of single-animal controls. Lobectomies were performed on 1 animal in each of 8 parabiotic pairs, and on 16 single control rats. Regeneration was based upon wet weights of liver estimated preoperatively, or measured at time of hepatectomy and at autopsy. Mitotic figures of liver tissue at time of lobectomy and at sacrifice were also studied.

Method. The range of body weight used in our rat group was from 125-225 g. The parabioses were performed on Anheuser-Busch rats, paired as closely as possible by weight and color. Successful parabioses were obtained with pairs of males and pairs of females; no attempts were made to pair animals of opposite sex. A median incision was made from the xiphoid process 3-4 cm caudad. Ligaments binding the left lateral lobe to the posterior lobe and median lobe to the diaphragm were severed. Branches of the hepatic artery and portal vein were ligated with No. 2 chromatized catgut, and the left lateral and median lobes removed *in toto*, with only slight loss of blood. The animals were then sutured in two layers. This operation was performed on one animal in each parabiotic pair and on the single animal control group. Animals were fed chow biscuits and water during the experiment, with 20% glucose added to the drinking water of the hepatectomized animals for 1 postoperative day. Lobectomies were done 5-6 days after the parabiosis operation, since cross-circulation is established by the fourth postoperative day(3). Nembutal, 35 mg/kg intraperitoneally, was used in the parabiosis operations, and ether in the lobectomies. About 200,000 units of pencillin subcutaneously or intramuscularly was administered daily for 2-3 days after every operation. At various intervals up to 14 days following lobectomy the parabiotics were killed by exsanguination, separated and each parabion weighed. The liver weight of each animal in the union was recorded and sections made. The sections were placed in Bouin solution within 6 minutes after removal from the body.

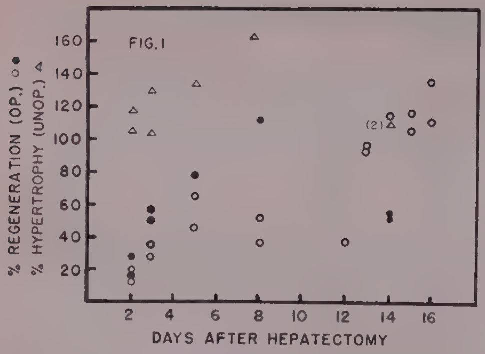
Results. The percentages of regeneration and of hypertrophy of the operated and unoperated livers, respectively, are plotted in Fig. 1. The percentage of regeneration of liver tissue in the lobectomized animals is that based on the fragment of liver removed,

---

1. Higgins, G. M., Anderson, R. M., *Arch. Path.*, 1931, v12, 186.

2. Franseen, A. M., Brues, A. M., Richard, R. L., *Endocrinology*, 1938, v23, 292.

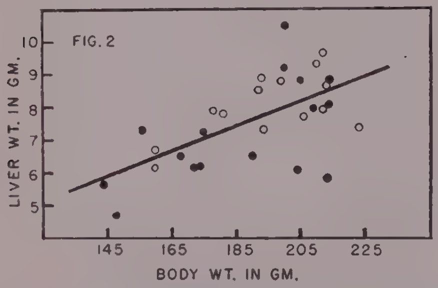
3. Moore, R. A., Hellman, L. M., Jacobius, H., *Arch. Path.*, 1942, v34, 196.



Open and closed circles represent the percentage of regeneration occurring in the single and parabiotic lobectomized animals, respectively. Regeneration is based upon the fraction of the liver removed as described in the following formula:

$$\% \text{ regeneration} = \frac{\text{Wt liver at autopsy} - \frac{1}{2} \text{ amount removed}}{\text{Amount removed}} \times 100$$

where ½ amount removed = amount remaining after lobectomy. Triangles represent the hypertrophy over normal occurring in the unoperated animal of the parabiotic union. The normal liver weight is based upon calculations made from Fig. 2.



Closed circles represent liver weights obtained by killing animals by exsanguination, followed by measuring the wet weights of their livers. Open circles represent liver weights determined by weighing the liver removed at time of lobectomy on the single animal control group, liver weight being 3/2 times the amount removed.

as explained in the legend. Thus, there has been 50% regeneration when the remaining liver tissue has increased so that half of the removed tissue has been replaced. The percentage of regeneration in our single animals follows fairly closely that obtained by Higgins. Regeneration in the earlier stage seems to occur at a faster rate in the parabiotic than in the control group, but since there are 2 exceptions at the 14 day period, the small size of the experimental group limits any generalization.

However, the liver weights found at autopsy in the unoperated parabions merit attention. Of the 8 parabiotic pairs used, the livers of the unoperated rats were without exception larger than normal. In fact, at the 8 day interval, one liver weight is recorded as being over 160% of the normal liver weight for that size rat. The curve in Fig. 2 was used to determine normal liver weights in the unoperated rats, since there was no other way of knowing the weights of these livers at time of union.

A microscopic study of the unoperated livers at time of sacrifice revealed no congestion, but hypertrophy of the parenchymal cells. Mitotic figures were counted in 10,000 or more liver cells taken at random in each of the unoperated livers and are tabulated as the number of mitoses per 100,000 liver cells.

	Days after hepatectomy				
	2	3	5	8	14
No. of mitoses per 100000 liver cells	150 200	220 60	64	350	6 4

Our counts were higher than those of Christensen(4) who obtained mitotic figure counts of 4, 30, and 33 per 100,000 liver cells on 3 parabions 3 days after hepatectomizing one in each pair. He does not report on weight changes. We observed no mitoses in 10,000 liver cells counted in each of 3 normal liver control slides.

In Fig. 2 a regression line of normal liver weights plotted against body weights in single unoperated animals was determined for our colony of animals. The plotting of points for this line was derived from two sources. Sixteen of the liver weights plotted were determined by weighing the animals, killing them by exsanguination, and measuring the wet weight of the livers. The determination of the other 14 liver weights was based upon the amount of liver removed from our single animal control group. Since 2/3 of the liver was removed at lobectomy, the total liver

---

4. Christensen, B. G., Jacobsen, E., *Acta Med. Scand.*, 1949, Supplementum 234, 103.

weight is 3/2 the weight of the liver removed. Fig. 2 shows no discrepancy between these 2 sets of points, which further substantiates Franseen's work. From the points plotted in Fig. 2, the slope of this line was derived from the formula,

$$M = \frac{\Sigma xy - n\overline{xy}}{\Sigma x^2 \quad n\bar{x}^2},$$

where the regression line passes through the mean point $(\bar{x}, \bar{y})$. The slope was found to be 0.0364 and the mean point (190.6, 7.613). The equation, therefore, is as follows: $y = 0.0364\ (x - 190.6) + 7.613$.

All but one parabiotic pair had lost various amounts of body weight by the time of autopsy. All of the rats were growing and the difficulty arose as to which body weight was to be used in Fig. 2 to determine what normal liver weight would have been at time of autopsy. We could not apply Jackson's data (5) as to age and duration of inanition to our experiments. The largest weight the rat had ever attained was therefore used to obtain predicted normal liver weight. If the smaller body weights were used, the ratio of liver weight at autopsy to calculated normal liver weight would be even greater than recorded.

To test the validity of the above comparison the ratio of liver to body weight both in normal animals and in unoperated parabiotics was determined. The mean ratio for the normal group was .040 ± .001* and for the experimental group .049 ± .003. The normal group contained 30 and the experimental group 8 animals. Applying the t-test for the comparison of two groups of different sizes, the probability that the difference occurred by chance is less than 2%.

Immediate postoperative mortality was very low in the parabiotic operations. However, many of the rats once in parabiosis died within the 5 days allowed between the first and second operations. These deaths were due to infections and incompatability. The overall mortality was more than 50%. Mortality due to lobectomies on both control and parabiotics was less than 1%.

Discussion. The parabiosis technic afforded a method of introducing a normal liver into the circulation of a partially hepatectomized rat. It was thus possible to observe the effect of this normal liver on one that regenerates, and the effect of the regenerating liver on the normal. Two factors must be present for growth to occur, (1) a response to a growth stimulus and, (2) nutritive materials without which growth cannot occur. Partially hepatectomizing a normal rat initiates a stimulus for liver growth. One cannot say whether this stimulus is due to the introduction of a new humoral factor, or to a humoral factor normally present but increased in amount due to failure of normal destruction by the liver. However, this experiment does indicate that the liver growth factor is humoral, and that a normal liver can respond to it just as does an operated liver, since the liver of the unoperated parabion responded to a substance which must have been humoral.

If the supply of nutrient material is a limiting factor in the rate of regeneration of the operated liver, the introduction of a normal liver into the joined circulation should bring about an increase in nutrient supply to the operated animal, with a resultant increase in the rate of its liver regeneration above that in the single hepatectomized animal. On the other hand, the presence of a normal liver might result in increased destruction of the growth-stimulating factor. Since in this experiment we cannot conclude that regeneration occurred faster in the lobectomized parabions than in the lobectomized single animals, both or neither of the above factors may be at work.

Summary. 1. When one rat of a parabiotic pair was partially hepatectomized, the unoperated liver of its partner increased in wet weight and in the number of hepatic cells. The growth stimulus causing this hypertrophy of liver tissue must have been a factor carried in the blood from the operated animal to the unoperated liver, and the latter responded to

---

5. Jackson, C. M., *J. Exp. Zool.*, 1915, v19, 99.

* Stand. dev. of the mean $= \sqrt{\frac{\Sigma x^2}{n\ (n-1)}}$.

this stimulus just as does an operated liver. 2. No significant difference was observed between the rates of regeneration of livers in lobectomized parabions and in lobectomized single animals.

Received February 16, 1951. P.S.E.B.M., 1951, v76.

Metabolites of Thyroxine.* (18595)

J. GROSS AND C. P. LEBLOND.
From the Department of Anatomy, McGill University, Montreal.

The recognition of thyroxine as the thyroid hormone(1,2) makes it important to study its products of metabolism. In the present work, the radioactive substances occurring in the body after administration of radio-thyroxine were detected by radioautography of paper chromatograms of tissues and excreta.

Methods. Radioactive L-thyroxine was prepared by biologically labeling its iodine as described earlier(3). This radio-thyroxine was injected subcutaneously into (a) 6 intact C_3H mice, (b) 3 thyroidectomized C_3H mice, and (c) 3 intact rats. Each animal of these experimental series was injected every 2 hours over a 12-hour period and sacrificed 13 hours after the last injection. The individual doses varied from 0.05 to 0.15 μg of thyroxine and contained from 1 to 5 μc of radioactivity. The purity of similar radio-thyroxine was found to average 92%(3). The nature of the 8% contamination was investigated by chromatographing the injection material according to the method described below. The contaminants were found to consist of traces of iodide and small amounts of an unidentified substance, hereafter referred to as compound No. 1. To remove these contaminants as completely as possible, a sample of radio-thyroxine was chromatographed, the radio-thyroxine spot cut out, eluted, and the elutant injected into one of the 3 thyroidectomized C_3H mice. This procedure successfully removed all of the iodide and all but minute traces of compound No. 1. For comparison purposes, 12 rats and 1 mouse were respectively injected with 100 and 20 μc of *radio-iodide* by the subcutaneous route. They were sacrificed 48 hours later. Liver, kidney, muscle and feces of each of the injected animals were homogenized in volumes of saline in the ratio of 10 cc per gram of tissue. Plasma and urine needed no homogenization and were used as such. All samples were first extracted with twice their volume of water-saturated butanol and then reextracted with an equal volume of the same solvent. These 2 butanol extracts were combined and concentrated to a small volume of *vacuo.* Aliquots were chromatographed on Whatman No. 1 filter paper along 2 dimensions and the resulting chromatograms were subsequently radioautographed. The technical details and the method used for the identification of the spots on the radioautographs have been reported elsewhere(2,4). Preliminary chromatographic runs yielded similar qualitative results for the starting materials (whole plasma, kidney homogenates in saline, etc.) and the corresponding butanol extracts. Since butanol extracts were easier to handle, they were adopted for routine work.

Results. The injection of *radio-thyroxine* gave similar results in intact and thyroidectomized animals. Plasma radioactivity was predominantly in the form of thyroxine (Fig. 1), but there were also small amounts of iodide and of an unidentified substance located to

---

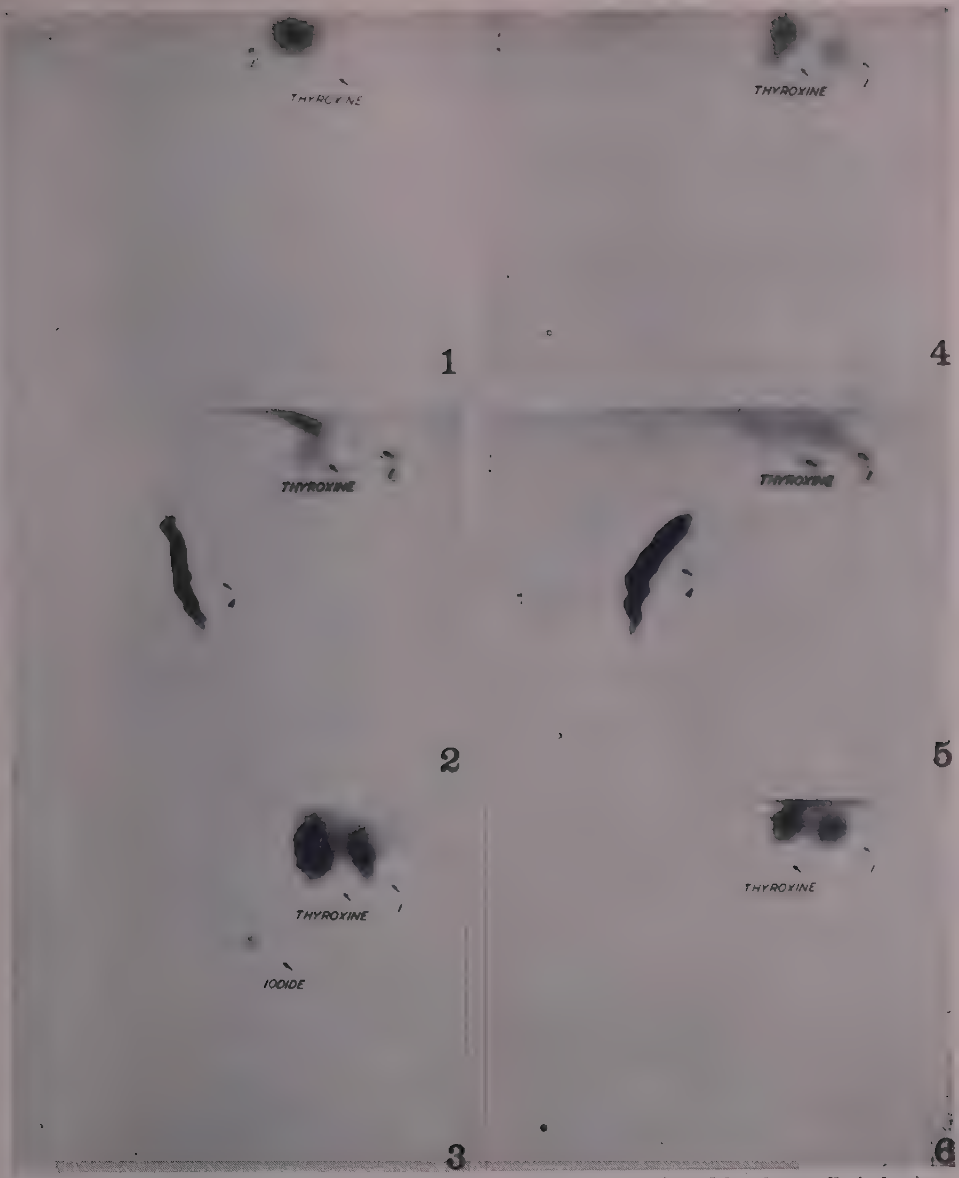
* This work was supported by a grant of the National Research Council of Canada.

1. Leblond, C. P. and Gross, J., *J. Clin. Endocrinol.*, 1949, v9, 149.

2. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J., *Science*, 1950, v111, 605.

3. Gross, J., Leblond, C. P., *J. Biol. Chem.*, 1950, v184, 489.

4. Gross, J. and Leblond, C. P., *Endocrinology*, 1951, in press.



Radioautographs of paper chromatograms. The origins are indicated by the small circles in the lower left hand corner.

FIG. 1. Plasma of C_3H mouse given labeled thyroxine over 24 hr period.
FIG. 2. Kidney of same animal.
FIG. 3. Feces of same animal.
FIG. 4. Plasma of C_3H mouse given radioiodide 48 hr previously.
FIG. 5. Kidney of same animal.
FIG. 6. Feces of same animal.

the right of thyroxine and referred to as compound No. 1. The kidney contained thyroxine, compound No. 1, traces of iodide, and an unknown compound—referred to as No. 4—which was located to the middle left of the radioautographs and was particularly abundant in mice (Fig. 2). The liver showed an intense thyroxine spot and moderate spots for iodide, compounds No. 1 and No. 4. Muscle and feces contained thyroxine, compound No. 1, and small amounts of iodide (Fig. 3), with the possibility of a faint spot corresponding to compound No. 4. The urine yielded chromatograms which differed from the others by the absence of thyroxine and compound No. 1, but iodide—the only substance identified with certainty—was present in large amounts.

Forty-eight hours after injection of *radio-iodide,* the picture was more or less similar to that obtained after radio-thyroxine administration. Thyroxine itself was found in plasma (Fig. 4), organs (Fig. 5), and feces (Fig. 6), but not in urine. Compound No. 1 had the same distribution (Fig. 2, 4 and 6). A large amount of compound No. 4 appeared in the kidney, especially in that of the mouse (Fig. 4). The urine contained very large amounts of iodide.

Discussion. After *radio-thyroxine injection,* 4 labeled substances were found: (1) thyroxine—in plasma (Fig. 1), organs (Fig. 2), and feces (Fig. 3); (2) iodide—mainly in urine(5,6); (3) compound No. 4—predominantly in the kidney (Fig. 2); and (4) compound No. 1—with the same distribution as thyroxine. Compounds No. 1 and No. 4 were different from all so far investigated iodine compounds of biological significance.

The possibility that these substances were artefacts due to the radiochemical effect of the β-rays from radio-iodine may be discounted, since small doses of radioactivity (less than 5 μc of radiothyroxine were used. Similarly, the possibility that the thyroid gland played a role in the formation of these substances may be dismissed, since the results obtained in thyroidectomized animals did not differ from those in intact controls. It was, therefore, concluded that the substances observed were metabolites of thyroxine and were formed in peripheral tissues.

The origin of compound No. 1 had to be especially considered. Since this substance was shown by chromatography to contaminate the radio-thyroxine administered to the animals, the spots for compound No. 1 found in chromatograms of tissues and feces might simply have been due to a retention of the injected contaminant. However, while these spots were much weaker than the thyroxine spots in the injection material, they were often just as intense in tissues. Thus, if they were due to a retention in the body of the compound No. 1 contaminating the injected thyroxine, this retention would have to be far more efficient than that of thyroxine. This did not seem to be the case, since both substances were equally excreted in feces (Fig. 3). Therefore, the alternative possibility, that is, that compound No. 1 was derived from thyroxine, provided a more satisfactory explanation for the large amounts of this compound found in tissues and feces.

Forty-eight hours after *radio-iodide injection* the same four substances—thyroxine, iodide, compounds No. 1 and 4—were found in tissues (Fig. 4 and 5) and excreta (Fig. 6). Presumably, the injected radio-iodide had first been transformed into radio-thyroxine by the thyroid; and this endogenous radio-thyroxine, following release into the circulation(1,3,6), had been metabolized to yield the other iodine-containing substances detected. The previous finding of compound No. 1 in thyroid tissue and plasma(4) raised the possibility that this substance, like thyroxine, was released from thyroglobulin and passed into the circulation to form a second thyroid hormone. However, the alternative possibility, namely, that compound No. 1 was a product of thyroxine metabolism occurring wherever thyroxine was present, even in the thyroid, would seem to be in better agreement with the results obtained with radio-thyroxine.

Summary. Following injection of I^{131} *labeled L-thyroxine* into intact and thyroidectomized mice and intact rats, tissues and excreta were investigated by radioautography of paper chromatograms of their butanol extracts. Four substances, namely thyroxine, iodide and two unidentified iodine containing substances referred to as compounds Nos. 1

---

5. Gross, J. and Leblond, C. P., *J. Biol. Chem.*, 1947, v171, 309.

6. Clayton, J. C., Free, A. A., Page, J. E., Somers, G. F., and Woollett, E. A., *Biochem. J.*, 1950, v46, 598.

and 4 were found. The same four substances were likewise found in organs and excreta taken from intact mice and rats injected 48 hours previously with *radio-iodide,* or in other words, after a time interval sufficient for endogenous production of labeled thyroxine in the thyroid and its subsequent distribution in the body. These findings suggest that thyroxine is metabolized to yield at least the two compounds Nos. 1 and 4 as well as iodide.

Received February 19, 1951. P.S.E.B.M., 1951, v76.

In vitro Inhibition of Radiophosphate Uptake and Growth of a Neurotropic Virus by 5-Chlorouridine.* (18596)

MAX E. RAFELSON, JR.,† HAROLD E. PEARSON, AND RICHARD J. WINZLER.‡

From the Departments of Biochemistry and Microbiology, University of Southern California School of Medicine and Los Angeles County Hospital, Los Angeles.

It has previously been reported(1-3) that infection of minced 1-day-old mouse brain with Theiler's GD VII strain of mouse encephalomyelitis virus increases the incorporation of $P^{32}O_4$ into the phospholipide and total protein-bound phosphate fractions. The increased turnover in the total protein-bound phosphate fraction was due primarily to the increased turnover of the ribonucleic acid fraction, the slower turnover of desoxyribonucleic acid phosphorus being unaffected by virus propagation(1). These experiments suggested that virus propagation was intimately associated with ribonucleic acid metabolism, and raised the question of what effects a nucleoside antagonist might have on virus propagation and nucleic acid metabolism. Previous studies have shown that 8-azaguanine had little effect on the growth of Theiler's virus in minced 1-day-old brain or on $P^{32}O_4$ metabolism of this system(3). Benzimidazole, however, inhibited virus growth and $P^{32}O_4$ uptake(3). 5-Chlorouridine has been shown to be a potent uridine antagonist in Neurospora(4). Preliminary experiments showed that 5-chlorouridine inhibited the propagation of Theiler's GD VII virus and led to the present experiments. In these experiments we have investigated the effects of 5-chlorouridine and of uridine, alone and in combination, on the uptake of $P^{32}O_4$ by the acid-insoluble fractions of minced 1-day-old mouse brain and on the propagation of Theiler's GD VII virus in this tissue.

Methods. The methods employed have been described in previous papers(1-3). Minced brain tissue, 40 to 60 mg, aseptically removed from 1-day-old mice was added to sterile 50 ml Erlenmeyer flasks containing 2.5 ml of Simms' solution containing 1 microcurie of $P^{32}O_4$. Uridine and 5-chlorouridine§ were added in concentrations of 3 mg per ml and 1 mg per ml of Simms' respectively. The pH was adjusted to 9 with dilute NaOH. The preparations were inoculated with virus or control supernatants, and brought to a final volume of 3 ml with Simms' solution. The flasks were closed with rubber stoppers and incubated 24 hours without shaking at 35°C in an atmosphere of air. At the termination

---

* This work was supported by a grant in aid from the National Advisory Cancer Council of the National Institutes of Health.

† Predoctoral Research Fellow of the National Institutes of Health.

‡ With the technical assistance of Dorothy Lagerborg and John N. Steward.

1. Rafelson, M. E., Winzler, R. J., and Pearson, H. E., *J. Biol. Chem.,* 1949, v181, 583.

2. Rafelson, M. E., Pearson, H. E., and Winzler, R. J., *Science,* 1950, v112, 231.

3. Rafelson, M. E., Pearson, H. E., and Winzler, R. J., *Arch. Biochem.,* 1950, v29, 69.

4. Fukahara, K., and Visser, D., *J. Biol. Chem.,* in press.

§ We are indebted to Dr. Donald Visser for the 5-chlorouridine.

of the incubation period, all preparations were tested for sterility and the virus titer determined as previously described(1,3,5). Studies were made of the uptake of radioactive orthophosphate into the organic acid-soluble, phospholipid (LP), and the various protein-bound phosphate fractions by procedures previously described(1). As the turnover and the distribution of phosphorus in the organic acid-soluble fractions of the present experiments were not significantly different from values previously reported(1), and neither of the compounds employed in this report exhibited any significant effect on these values, the data for this fraction have been omitted in this report. The protein-bound phosphorus was fractionated as follows:¶ The trichloroacetic acid-insoluble fraction after removal of lipides with ethanol-ether and chloroform-methanol was treated with 5 ml of 1N NaOH (phosphate-free). After a sample of the clear solution was removed for the determination of total protein-bound phosphorus, the solution was acidified with 0.1 volumes each of concentrated HCl and 50% trichloroacetic acid to precipitate desoxyribonucleic acid (DNA) and residual protein (RP). The acid-supernatant contained ribonucleic acid (RNA) and inorganic phosphate (PIP), presumably liberated from phosphoprotein during treatment. Contaminating inorganic phosphate will also be found in this fraction. The PIP fraction was separated from the RNA by the method of Delory(6). The DNA was extracted from the RP by two 15 minute treatments with 5 ml of 5% trichloroacetic acid at 90°C. This method corresponds closely to that developed independently by Furst *et al.*(7). Previous experiments(1) have shown the nucleic acid phosphorus values obtained in this manner were in good agreement with those calculated from pentose and desoxypentose determinations. The chemical analysis for phosphorus was carried out by a modification of the method of Fiske and Subbarow(8) in which ascorbate was used as a reducing agent and a heating period was employed for color development and stabilization. Radioactive samples were prepared in ¼-oz. tin ointment dishes and counted with a thin mica-window Geiger-Muller counter tube. A minimum of 3,000 counts per sample was taken and all counts were at least 3 times background.

Experimental. Table I gives data for the uptake of $P^{32}O_4$ in a 24-hour incubation period into the LP, RNA, DNA, RP, and PIP phosphate fractions of noninfected and virus-infected minced 1-day-old mouse brain as influenced by the presence of uridine and 5-chlorouridine in concentrations of 3 mg and 1 mg per ml of Simms' respectively. Values are expressed as relative specific activity, *i.e.*, the specific activity of the fraction relative to the specific activity of the inorganic phosphate fraction. It can be seen from Table I the presence of virus stimulated the uptake of $P^{32}O_4$ into the LP and RNA fractions as previously reported. The turnover of the other fractions was unaffected by the presence of virus.

Addition of 5-chlorouridine not only eliminated the virus effect on the RNA fraction but reduced the uptake below the values for the noninfected, control tissue. Curiously the stimulating effect of virus on the LP fraction was still present. No effects were noted on any other fractions. It is of interest to note that 5-chlorouridine had no effect on any of the fractions of the control tissue. Along with the inhibition of RNA turnover, virus propagation is also inhibited by 5-chlorouridine as evidenced by a reduction in titer from 50,000 to 1,000. In other experiments virus titers were determined after flasks had been incubated for 72 hours. The titers were 100,000 for control tissue with or without uridine, 50,000 with 5-chlorouridine and 100,000 with uridine plus 5-chlorouridine.

These observations suggest that RNA metabolism associated with virus propagation is more sensitive to 5-chlorouridine than is the

---

5. Pearson, H. E., *J. Immunol.*, 1950, v64, 447.

¶ In a previous publication(1) this portion of the fractionation procedure was erroneously reported due to the deletion of a sentence.

6. Delory, G., *Biochem. J.*, 1938, v32, 1161.

7. Furst, S. S., Roll, P. M., and Brown, G. B., *J. Biol. Chem.*, 1950, v183, 251.

8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

TABLE I. Effects of 5-Chlorouridine and Uridine on $P^{32}O_4$ Uptake by Acid-Insoluble Fractions of Minced 1-day-old Mouse Brain and on Propagation of Theiler's GD VII Virus. (Three independent series of experiments).

		Relative specific activity*			
Fraction		No addition	5-Chloro-uridine	Uridine	5-Chloro-uridine + uridine
LP	No virus avg	3.02	3.17	3.05	2.87
	Range	2.8-3.2	3.1-3.3	3.0-3.2	2.8-2.9
	Virus avg	3.87	3.9	3.88	3.89
	Range	3.6-4.1	3.8-4.1	3.8-4.0	3.8-4.0
RNA	No virus avg	2.78	2.42	2.51	2.63
	Range	2.6-3.0	2.2-2.8	2.4-2.6	2.6-2.7
	Virus avg	4.07	1.9	4.15	2.96
	Range	3.6-4.6	1.8-2.0	4.0-4.3	2.9-3.0
DNA	No virus avg	.64	.43	.57	.59
	Range	0.5-0.8	0.4-0.5	0.5-0.6	0.5-0.7
	Virus avg	.66	.55	.62	.66
	Range	0.5-0.9	0.3-0.7	0.6-0.7	0.6-0.7
PIP	No virus avg	4.7	4.31	4.14	4.14
	Range	4.3-5.5	4.2-4.9	4.0-4.3	4.0-4.3
	Virus avg	4.16	4.22	4.76	4.13
	Range	4.1-4.3	4.0-4.4	4.6-4.9	4.1-4.2
RP	No virus avg	.93	.95	1.16	1.1
	Range	0.7-1.3	0.7-1.1	1.1-1.2	0.9-1.3
	Virus avg	1.12	1.09	1.08	1.05
	Range	0.8-1.4	0.9-1.5	1.0-1.1	1.0-1.1
Virus titer†		50000	1000	50000	5000

$$\text{* Relative specific activity} = \frac{\text{counts}/\gamma\ \text{P in fraction}}{\text{counts}/\gamma\ \text{P in inorganic fraction}} \times 100.$$

† Virus titer is expressed as the highest dilution of the supernatant solution that would kill at least half of a group of 7 mice after intracerebral injection of each with 0.03 ml.

normal metabolism of RNA. Uridine by itself had no measurable effect on virus propagation or on the uptake of $P^{32}O_4$ into any of the tissue fractions of either the control or infected tissue. Uridine, however, partially overcame the inhibiting action of 5-chlorouridine on virus propagation and on the turnover of $P^{32}O_4$ in the RNA fraction of the virus-infected tissue.

In Table II the data for the distribution of phosphorus in the various tissue fractions from the experiments in Table I are given. As previously reported(1) the presence of virus increases the content of RNA and decreases the content of DNA. Addition of 5-chlorouridine inhibits this virus effect and reduces the RNA content to that of the noninfected tissue. The DNA content of the control and infected tissue may possibly increase slightly in the presence of 5-chlorouridine, although the magnitude of this effect is too small to establish this with certainty. Uridine appears to have no effect on the distribution of phosphorus in any of the fractions of the control or virus-infected tissues. Uridine in the presence of 5-chlorouridine partially restores the virus effect on the RNA content as shown by a rise from 15.8 to 18.3 and partially reverses the virus inhibiting action of 5-chlorouridine.

Discussion. It has been shown that propagation of Theiler's GD VII virus in minced 1-day-old mouse brain is associated with both an increased turnover and an increased net amount of RNA. The finding that virus propagation is inhibited when these effects of virus propagation are prevented suggests that RNA plays an intimate role in the propagation of Theiler's GD VII virus in minced 1-day-old mouse brain. Since 5-chlorouridine inhibits these virus effects on the RNA fraction, while having no effects on any other tissue fractions examined, possibly 5-chlorouridine is acting as a specific inhibitor

TABLE II. Effects of 5-Chlorouridine and Uridine on the Distribution of Phosphorus in the Acid-Insoluble Fractions of Minced One-day-old Mouse Brain. (μg of P per 100 mg of tissue).

Fraction		No addition	5-Chloro-uridine	Uridine	5-Chloro-uridine + uridine
LP	No virus avg*	35.7	34.4	38.5	38.3
	Range	35-36	34-35	36-40	38-39
	Virus avg	35.3	36.6	38.2	33.2
	Range	34-37	34-39	36-40	32-35
RNA	No virus avg	16.0	16.0	16.4	15.2
	Range	15-17	15-17	16-17	15-16
	Virus avg	19.9	15.8	20.2	18.3
	Range	19-21	15-17	20-21	18-19
DNA	No virus avg	12.4	14.5	13.0	12.5
	Range	11-13	13-15	12-14	12-13
	Virus avg	10.8	12.3	10.6	10.3
	Range	10-11	11-14	10-11	9-11
PIP	No virus avg	6.2	5.2	6.3	6.5
	Range	4-7	5-6	6-7	5-7
	Virus avg	5.9	6.1	4.8	5.8
	Range	5-7	5-7	4-5	5-6
RP	No virus avg	3.2	2.9	2.6	2.3
	Range	2-4	2-4	2-3	2-3
	Virus avg	3.7	2.7	2.5	2.6
	Range	3-6	2-3	2-3	2-3

* Avg of 3 experiments.

of RNA metabolism associated with virus propagation in this system.

Summary. 5-Chlorouridine inhibits the propagation of Theiler's GD VII virus and the uptake of $P^{32}O_4$ by the ribonucleic acid fraction of minced, 1-day-old mouse brain but had no effects on other tissue fractions studied. Uridine partially reverses the inhibiting effects of 5-chlorouridine on virus propagation and on the uptake of $P^{32}O_4$ by the ribonucleic acid fraction. Virus propagation has been shown to be associated both with an increased turnover and increased net amount of ribonucleic acid phosphorus.

Received February 19, 1951. P.S.E.B.M., 1951, v76.

A Substance Occasionally Found as a Major Ninhydrin-Reacting Component of Urine.*† (18597)

KAY FINK.

From the Department of Investigative Medicine, Veterans Administration Hospital, Long Beach, Calif. and the Department of Biophysics, University of California Medical School, Los Angeles

A ninhydrin-reacting substance has been observed frequently on 2-dimensional paper chromatograms prepared from the urines of many subjects, which appears not to match any of the 60 ninhydrin-reacting substances given in the "map of spots" by Dent(1), most of which were available for direct comparison, as well as a number of additional compounds available for checking. This unidentified compound falls between alanine and proline

---

* Aided by a grant from the University of California Cancer Research Fund.

† Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Dent, C. E., *Biochem. J.*, 1948, v43^2, 169.

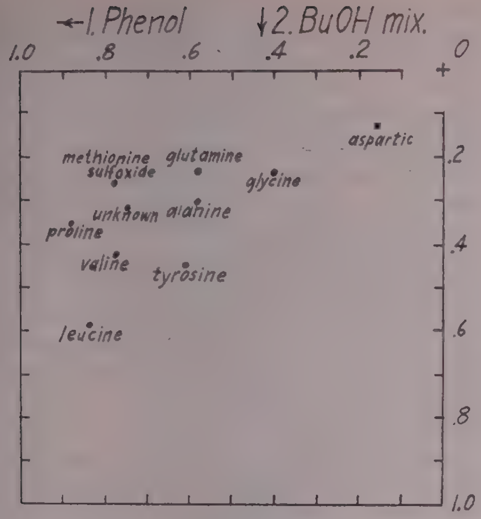


Fig. 1.

A 2-dimensional chromatogram showing the location of the unidentified substance and some common amino acids for orientation: Phenol saturated with water was used as the first chromatographing solvent and the upper layer of a mixture of tertiary butyl alcohol, secondary butyl alcohol, and water (1:5:5.6) as the second solvent.

when phenol is used as the chromatographing solvent, and between methionine sulfoxide and valine when the upper phase of either collidine, lutidine, and water (1:1:2) or tertiary butyl alcohol, secondary butyl alcohol, and water (1:5:5.6) is used as the solvent. Its position on a 2-dimensional chromatogram is shown in Fig. 1. It falls close to the position taken by α-amino-n-butyric acid on a 2-dimensional chromatogram, and judging from the frequency with which we have observed this compound on urine chromatograms (using 25 μl samples of first morning urine specimens from a large number of normal and pathological subjects) and the extreme rarity of any spot in the position of α-amino-n-butyric acid on these chromatograms, it is conceivable that others may have mistaken it for α-amino-n-butyric acid. The position of the unidentified compound in routine 2-dimensional chromatograms is close to the positions occupied by a number of known compounds, including sarcosine, pantonine, α-amino-ε-hydroxycaproic acid, α-amino-isobutyric acid, α-amino-n-butyric acid, γ-amino-n-butyric acid, σ-amino-n-valeric acid, and homocitrulline, but obvious differences can be shown with side by side comparison of R_f values in a wide variety of solvents. There remains the rather remote possibility that the unidentified substance in the urine might be a relatively stable salt, complex, or derivative of one of the known compounds with which it has been compared chromatographically or that it is a common substance previously known to be present in the urine but never reported as a ninhydrin-reacting constituent located on paper chromatograms. Attempts to isolate the substance from large volumes of urine in order that standard chemical procedures may be employed in its characterization have not as yet been successful but progress is being made in this direction.

A crude preparation of the unidentified compound has been obtained from 50 ml of urine by sectioning paper chromatograms. Urine from a leukemic patient who excreted the compound under investigation in high concentration was applied as a single line across the top of 10 sheets of filter paper, 22″ x 18½″. Phenol, with no ammonia in the atmosphere, was used as the chromatographing solvent, and when the boundaries were a few centimeters from the bottom the sheets were removed, air-dried, and examined in ultra violet light. All fluorescent bands were marked to serve as a guide for locating irregularities in the travel of bands on these overloaded papers. Two narrow strips, about one-half inch wide, were cut from each sheet of paper perpendicular to the line along which the urine was streaked originally and at about one-half of the distance from the center to each side of the sheet of paper. These strips were sprayed with ninhydrin in the usual fashion and the position of the unidentified compound could then be detected by the color reaction. A combination of the position of the ninhydrin-positive bands on the sprayed strips and the fluorescent bands were then used to locate the substance on the unsprayed sheets, and strips containing the unknown, together with some overlapping substances, were cut from each of the sheets of paper. A rectangular piece of filter paper, about 4 inches long, was sewed by machine to one end of each strip, and a triangular

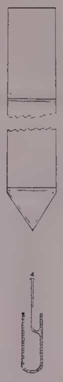


FIG. 2.
Arrangement for collecting the substances from a strip of filter paper in a small volume of solvent: A strip of filter paper was sewed to the top for insertion in the solvent trough, and a triangular piece of filter paper was sewed to the opposite end of the paper, with threads left at the apex for tying to a glass collection bucket.

piece of filter paper was sewed to the opposite end of each strip. Long threads were left at the point of the triangular piece of paper, and these were tied to a small glass bucket, as shown in Fig. 2. The top of the strip was then supported in the trough of an all glass apparatus used for one-dimensional chromatography, and water was used as the solvent. The volume of water placed in the trough was small, so as to permit slow travel down the strip. When the water, which now contained substances washed from the strip, reached the triangular point of the paper, it followed down the threads and was collected in the bucket. When the bucket was full, the strip was removed and the sample collected from the bucket. The strip was allowed to dry, inserted in the trough again, and a second wash collected. Each strip cut from the original 10 papers was handled in this fashion, and the combined washings were pooled, streaked across eight large sheets of filter paper, and this time a mixture of tertiary butyl alcohol, secondary butyl alcohol, and water (1:5:5.6) was used as the chromatographing solvent. The sheets were then treated as previously, using fluorescent bands and small strips sprayed with ninhydrin as guide lines for cutting out the unidentified compound. The strips containing the unknown were sewed, buckets were attached, and the substance washed off with water. The pooled collections were streaked across one large sheet of filter paper which had been previously washed to remove the peptide contaminants(2), and chromatographed in phenol with ammonia present in the atmosphere. The unknown substance was removed as before, and streaked again on a sheet of paper previously washed, and chromatographed in collidine-lutidine-H_2O (1:1:2). The section of the paper corresponding to the compound in question was washed into the attached bucket, and this collection contained the unknown substance, with only faint traces of other ninhydrin-reacting compounds. After adjusting the volume to 7 ml, the intensity of the ninhydrin color produced by this fraction, using 5 μl for a 2-dimensional chromatogram, was roughly equivalent to that found with the common amino acids, using 5 μl of 0.01 N solutions. Probably around 10 mg of the material was obtained by the streaking technic, but although it was relatively free of other ninhydrin-reacting substances it showed no tendency to crystallize when subjected to the usual crystallization procedures.

This preparation has been used routinely as a marker for comparison with other amino acids available for study, for comparison with other urines showing the spot on 2-dimensional chromatograms, for following isolation procedures on a larger scale by other technics, and to determine some of the chemical properties of the compound.

The compound is stable to acid hydrolysis (6 N-HCl, 100°C, 24 hours). Its R_f value in phenol is not affected by ammonia or by hydrochloric acid in the atmosphere, nor is its rate of travel in phenol and collidine-lutidine affected by H_2O_2. The diazo test with the Pauly reagent(3) is negative, the bleaching reaction with potassium iodoplatinate for sulfur-containing amino acids described by Winegard and Toennies(4) appears to be negative, the benzidine-ammonium molybdate

---

2. Wynn, V., *Nature*, 1949, v164, 445.

3. Pauly, H., *Hoppe-Seyl. Z.*, 1904, v42, 508.

4. Winegard, H. M. and Toennies, G., *Science*, 1948, v108, 506.

color reaction for phosphate(5) is negative. The substance cannot be extracted with ether from a 0.3 N NaOH solution, which suggests it is not an amine. When the filter paper is brushed with basic cupric carbonate before the substance is chromatographed, using the technic described by Crumpler and Dent(6) as a distinctive test for α-amino acids, the ninhydrin test is not negative as for α-amino acids, but the ninhydrin color obtained is no longer the typical purple color, but has a brownish hue. This test has been carried out several times, using α-amino-n-butyric acid, γ-amino-n-butyric acid, and the unknown on the same strip. The α-amino acid consistently gave no color with ninhydrin, the γ-amino acid gave the typical color reaction, and the unknown gave an altered color. A somewhat similar reaction to copper dusting is shown by some amines and basic amino acids, but since the chromatographic behavior of the unidentified compound does not suggest a strongly basic nature, interpretation of the results of the copper test is equivocal.

Whatever the compound may be chemically, it is very interesting biologically. It has been observed occasionally in urines from some of our group of normal subjects composed of resident physicians, nurses, and laboratory personnel, and more frequently in urines from patients with certain pathological disorders. In many of the chromatograms, it is seen as a very light spot, but its presence is not restricted to cases of aminoaciduria or to highly concentrated urines. Occasionally it is seen to be the most intense spot on the chromatogram, and in a few cases, the only other ninhydrin-reacting substances detected were glycine, alanine and glutamine. It has been found in the urines of some of the patients examined with schizophrenia, epilepsy, various circulatory diseases, cerebral palsy, poliomyelitis, etc., but the highest concentrations have generally been observed in cases of malignancy. Chromatograms have been run on some patients with cancer over an extended period of time, and the excretion of the substance appears to be affected by some therapeutic regimes. In leukemia, for example, it may be reduced sharply in concentration or disappear with remission. Further data on the frequency of occurrence and the concentration of the compound in urine are being collected, and the results, together with information on the therapy and clinical state of individual subjects, will be reported.

Summary. An unidentified ninhydrin-reacting substance has been observed frequently on 2-dimensional paper chromatograms, prepared with urine from a wide variety of subjects, both normal and pathological. High concentrations are frequently found in the urine of patients with malignancy, and from results on some of the patients followed over an extended period of time, its concentration appears to be affected by certain therapeutic regimes. In leukemia, it may be reduced sharply in concentration, or disappear with a remission. A crude preparation has been obtained from urine by paper chromatography, and some of its properties investigated.

The author is indebted to Dr. W. Shive for a sample of pantonine, to Dr. A. C. Kurtz for homocitrulline, to Dr. L. J. Reed for the γ-amino-butyric acid, and to Dr. R. Gaudry for the α-amino-ϵ-hydroxycaproic acid. The writer wishes to thank Mrs. Frances Davis for technical assistance during the course of this investigation and Dr. R. B. Henderson, Dr. Phillip West, and Dr. R. M. Fink for their interest in this study.

Received February 20, 1951. P.S.E.B.M., 1951, v76.

---

5. Feigl, F., *Qualitative Analysis by Spot Tests*, Nordemann Publishing Company, Inc., New York (1937).

6. Crumpler, H. R. and Dent, C. E., *Nature*, 1949, v164, 441.

Growth of Neurotropic Viruses in Extraneural Tissues IV. Poliomyelitis Virus in Human Testicular Tissue *In vitro*.* (18598)

WAYNE M. SMITH, VELMA C. CHAMBERS, AND C. A. EVANS.

From the Department of Microbiology, University of Washington School of Medicine Seattle, Washington.

Multiplication of the Lansing strain of poliomyelitis virus has been demonstrated in tissue cultures of human embryonic tissue (1,3) and of human foreskin(2). Evidence suggesting limited multiplication in human placenta in 2 of 13 tests has also been reported (3). The Brunhilde strain has been maintained in human embryonic skin and muscle cultures for 73 days and in human embryonic brain cultures for 39 days(2).

In this paper it will be shown that poliomyelitis virus of the Lansing and Hof. strains multiplied extensively in human testicular tissue *in vitro*. In experiments with other human tissues our results have been largely, but not completely, negative. In a preliminary report of this work, a more extensive review of pertinent literature will be found (4).

Materials and methods. Source of tissue. The testes used in these experiments were from men 57 to 93 years old, most of whom were suffering from carcinoma of the prostate gland. Some had received estrogen therapy prior to operation. Histologically the testes varied considerably. In most of them, spermatogenesis was reduced and in some cases it was apparently absent.

Viruses employed. The Lansing virus used in these experiments was received from Dr. John F. Enders in December, 1948. It had been passed in mice 2 times in our laboratory prior to use in these experiments. For preservation of the virus, suspensions of infected CNS of mice were stored in the dry ice chest.

The Hof. virus, which we received from Dr. A. B. Sabin in May, 1949, was isolated from stools and throat swabs from a patient in Cincinnati in 1947 and has since been found to belong to the Brunhilde group of poliomyelitis viruses. The virus had been passed through rhesus monkeys 3 times before we received it. Suspensions of the infected spinal cords from the cynomolgus monkeys representing the 1st and 2nd passages in our laboratory were preserved in the dry ice chest until used in these experiments.

Tissue culture methods. Promptly after the testes were removed from the patient, small fragments were cut from the central portion of the organ, placed in warm tissue culture medium and minced to approximately 1 mm to 3 mm in size. About 1 g of the testicular material in 30 cc of medium was transported to the laboratory in a portable incubator where the fragments were minced again with a Bard Parker scalpel until they were 1 mm or less in diameter. The tissue was washed with medium several times during the mincing process in an attempt to reduce the amount of antibody carried over with the tissue. Approximately 0.02 to 0.04 ml of a heavy suspension of minced tissue was transferred by means of a graduated pipette to the center of a perforated cellophane disc in each of 3 50-ml Erlenmeyer flasks. Virus was promptly added as 0.4 ml of a centrifuged 10% suspension of infected tissue in tissue culture medium. A period of 5 minutes was allowed to elapse after which 3.6 ml of medium were added, producing a tissue-to-fluid ratio of approximately 1:100. The tissue culture medium consisted of bovine serum ultrafiltrate[†] diluted with 3 volumes Hank's bal-

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Enders, J. F., Weller, T. H., and Robbins, F. C., *Science*, 1949, v109, 85.

2. Weller, T. H., Robbins, F. C., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 153.

3. Milzer, A., Levinson, S. O., Vanderbrom, K., and Adelman, P., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 136.

4. Smith, W. M., Chambers, V. C., and Evans, C. A., *Northwest Medicine*, 1950, v49, 368.

[†] The serum ultrafiltrate and Hank's solution were obtained from Microbiological Associates, Coral Gables, Fla.

anced salt solution. It contained phenol red and 100 units of penicillin and 100 μg of streptomycin per ml. The atmosphere in the flasks was flushed with 5% CO_2 and 95% air. The flasks were closed with rubber stoppers and loose aluminum caps were put over the top to minimize contamination of the lip of the flask. The cultures were placed in the incubator at 36°C. The medium in the tissue cultures was changed regularly at intervals of 3 or 4 days. At this time 3.6 ml of fluid were removed and replaced by an equal volume of fresh medium. Tests for virus were made by injecting the harvested fluid (pooled from 3 flasks) intracranially in amounts of 0.03 ml into mice or 0.5 to 1.0 ml into monkeys. The inoculum contained a minimum of cells and no attempt was made to free virus from cells. Transfer of virus to new tissue was made by adding 0.4 ml of pooled tissue culture fluid from flasks of previous cultures to the tissue of the new culture. Frequently the fluid was frozen in a sealed glass tube in the dry ice chest for some time prior to being used for transfer or for virus titrations. There was no evidence of fall in pH values (change in color of phenol red) of the tissue culture fluid during the periods of incubation. Only new pyrex glass flasks were used. The flasks were washed with Ivory soap and rinsed 5 times with tap water and 5 times with distilled water. The cellophane, perforated with the Beckley OC design, was obtained from the Tissue Culture Association and cut into discs slightly smaller than the bottoms of the flasks. These were washed with 3 changes each (10 minutes per "change") in ether, absolute alcohol, acetone, distilled water, and glass distilled water. They were then placed in clean flasks which were autoclaved and stored until used.

Multiplication of Lansing virus in human testicular tissue. Lansing poliomyelitis virus was inoculated onto human testicular tissue in our 1st experiment on November 29, 1949. As shown in Fig. 1, it was passed through 8 successive tissues. The calculated dilution of the original inoculum was 10^{-23}. In no instance did loss of virus necessitate going back to an earlier specimen to resume the passage of virus. It is estimated that each time fluid was changed, a 10-fold dilution of virus occurred. Evidence for the validity of this method of calculation is found in Fig. 1. With a few exceptions, every specimen of fluid removed in the periodic change of medium was tested for infectivity. Results showed that the virus content gradually increased throughout the period of incubation up to the time transfer was made to new tissue. In all cases, the amount of virus removed was at least equal to that expected on the basis of simple dilution, and in most cases it was 10 times that amount. Daily tests of virus in the period beginning November 29 provide further evidence for the continuing presence of virus in fluid and support this method of calculation.

Identity of the virus. Observations were made periodically in order to check the identity of the virus during the course of these experiments. Cultures for bacterial contamination were consistently negative. The wide range in incubation period in test mice, from 3 to 30 days, is characteristic of Lansing virus. Mice that were observed while showing signs of illness almost invariably had paralysis of the front legs, the pattern of paralysis known to be characteristic of Lansing virus. Periodic checks made for ether resistance of the virus were always positive. Intramuscular injection of mice failed to produce visible infection. The titers of virus in the CNS of mice succumbing in tests of tissue culture fluid were all in the range expected with Lansing virus. No infectious agent was detected by intracranial injection of mice with suspensions of spleens from mice infected with tissue culture fluid. Extensive tests were made to check the identity of virus in the 8th serial passage representing estimated dilutions of 10^{-23} of the original inoculum. Evidence of the sort described above was obtained. In addition pathogenicity for monkeys was checked. The undiluted tissue culture fluid, treated with ether overnight, induced typical poliomyelitis in 2 injected monkeys. A 10^{-1} dilution of the same fluid produced poliomyelitis in 2 injected monkeys; neither of the 2 monkeys injected with a 10^{-2} dilution was infected. In 3 other experiments, Lansing virus has been maintained

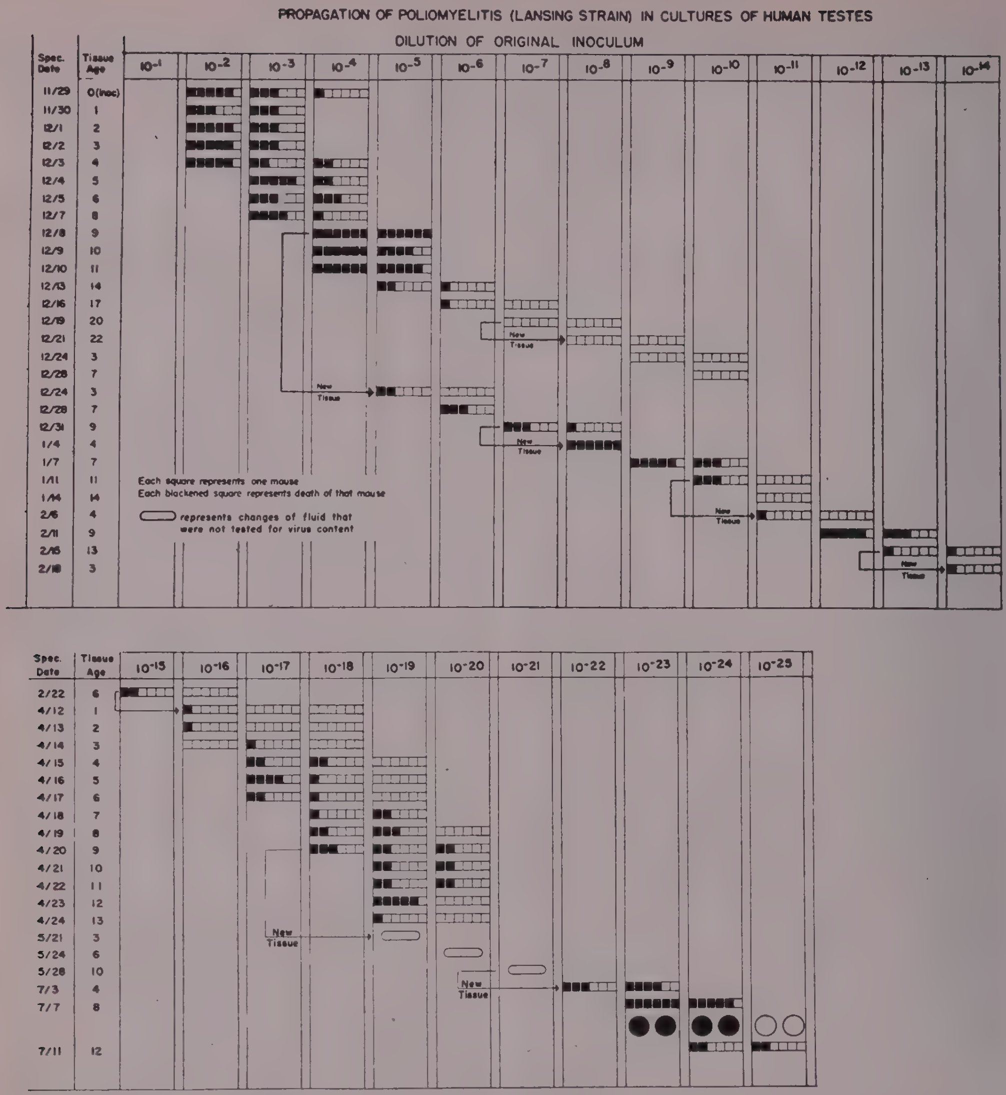


FIG. 1.

Tests for virus were made by intracranial injection of mice in groups of 6. Undiluted tissue culture fluid, fluid diluted 10-fold and in some cases fluid diluted 100-fold were used. The first horizontal line represents the titration of the virus used to inoculate tissue cultures on 11/29. At intervals of 3 or 4 days, 90% of the fluid was removed from each culture and replaced by fresh culture fluid. A 10-fold dilution of the original inoculum resulted each time fluid was changed and each time a transfer was made to a new culture.

Spec. date = date specimen was removed from tissue culture.

Tissue age = number of days the tissue in this culture had been maintained *in vitro*. In each case, transfer of infected fluid was made to new tissue on the day the new tissue was first placed in tissue culture medium.

Circles refer to monkeys inoculated. If infection occurred, the area inside the circle is black.

through 3, 4, and 5 successive tissue cultures of human testes with calculated dilutions of 10^{-8}, 10^{-13}, and 10^{-16} of the original inoculum. In each of these experiments the virus was present in the last passage tested. That is, in none of them was the virus lost due to failure to multiply. In a 5th experiment, Lansing virus was inoculated into a testicular tissue culture and was carried through a 2nd tissue with tests at 3- or 4-day intervals. Virus decreased markedly in the first few days and in all tests after this time only 1 or 2 mice succumbed in each group of 6 injected with tissue culture fluid. However, subinoculations from mice succumbing in these tests proved they were infected with virus having the characteristics of Lansing virus (ether resistance, incubation period, initial paralysis of front legs). This culture has been carried to a calculated dilution of 10^{-7} of original inoculum. The virus evidently maintained itself at a level insufficient to give a titer of 1 LD_{50} in the supernatant fluid. The same appeared to be true at some stages in the experiment represented in Fig. 1.

Multiplication of Hof. Virus in human testicular tissue. The Hof. strain of poliomyelitis virus was first inoculated into tissue cultures of human testicular tissue on December 31, 1949. Since that time, it has been passed through 7 successive tissue cultures. The calculated dilution of original inoculum was 10^{-21} in the 7th tissue culture. Since this virus will not infect mice, tests for virus have been much less frequent than in the experiments with Lansing virus. Four specimens representing the 1st, 2nd, 3rd, 5th and 7th passages were tested. Of the 13 monkeys injected with various specimens of undiluted tissue culture fluid, 1 escaped infection. Of the 19 monkeys injected with 1:100 dilutions of tissue culture fluid, 15 were infected. Of 9 monkeys injected with 1:1000 dilutions of tissue culture fluids, 3 were infected. Since the tissue-to-fluid ratio was 1:100 in these cultures, the titer of virus, in terms of tissue present, was between 10^{-4} and 10^{-5}. In other words, it equalled the amounts of virus obtained in equivalent amounts of infected monkey spinal cords. A pool of specimens removed at 6 or 7 days from cultures of 3 different tissues in this series infected 4 of 4 monkeys injected with a 10^{-2} dilution and 1 of 4 injected with a 10^{-3} dilution. A similar pool of specimens removed at 13 or 14 days from 3 cultures infected 2 of 4 animals injected with the 10^{-2} dilution and 1 of 4 injected with the 10^{-3} dilution.

Identification of this virus after passage in tissue cultures was based on the following evidence, all demonstrated with virus in the 7th passage in tissue culture:

1. The infective agent was ether resistant.
2. Intracranial inoculation of mice with material infectious for monkeys failed to cause illness of the mice.
3. Nearly complete flaccid paralysis of voluntary muscles of all 4 limbs developed in most infected monkeys; less severe involvement occurred in a small proportion of animals.
4. Typical histopathology of poliomyelitis was regularly found in spinal cords of infected monkeys.
5. Surviving monkeys were resistant to intracranial challenge with Hof. stock virus suspension representing more than 100 LD_{50}.

In 2 other experiments Hof. virus was maintained through 2 and 4 serial passages and virus was demonstrated in tissue culture representing 10^{-7} and 10^{-13} dilutions of the original inoculum. In no case did this virus fail to multiply in a tissue culture of human testicular tissue.

Discussion. It is evident that poliomyelitis virus of the Lansing and Hof. strains has multiplied extensively in tissue cultures of human testicular tissues. Our largely negative results obtained with other human tissues have been briefly summarized elsewhere(4).

Attempts to cultivate the viruses of herpes simplex and western equine encephalitis in tissue cultures of the same tissues used in experiments on poliomyelitis were largely unsuccessful except in testicular tissue. With the latter herpes virus grew; western equine encephalitis was not tested in this tissue. It seems probable that when suitable tissue culture conditions are defined, these 2 viruses and possibly poliomyelitis virus will multiply in some of the human tissues with which we have had negative results up to this time.

The kind or kinds of cells which support the growth of virus in testicular tissue cultures have not as yet been determined in spite of considerable effort to do so. Germinal cells degenerated rapidly in tissue cultures whether infected or not. Some of the testes in our series had markedly depressed germinal cell activity *in vivo*. Therefore it was probably not the germinal cells that were infected. The Sertoli cells, the interstitial cells of Leydig, and the fibroblasts remain to be considered. The susceptibility of interstitial cells to certain other viruses injected into the intact testis has been well demonstrated.

The capacity of testicular tissue from monkeys, bulls, hamsters, guinea pigs and mice to support multiplication of Lansing virus *in vitro* has been explored to a limited extent. At the present time it appears that Lansing virus has not multiplied in any of these experiments except possibly those in which tissue of cynomolgus monkeys' testes were employed.‡

Summary. The Lansing and Hof. strains of poliomyelitis virus multiplied extensively in tissue cultures of testes from men 57 to 93 years old, most of whom had carcinoma of the prostate gland. Lansing virus was carried through 8 successive tissue cultures and Hof. virus was carried through 7 cultures. Several less extensive series were completed with each virus. The amount of Hof. virus in these tissue cultures per milligram of tissue was approximately the same as was obtained in infected monkey spinal cords.

---

‡ In experiments completed after this paper was submitted for publication poliomyelitis virus multiplied readily in tissue cultures of testicular tissue from both rhesus and cynomolgus monkeys.

Received February 20, 1951. P.S.E.B.M., 1951, v76.

A Metabolic Intermediate of Pamaquine from Chickens. (18599)

EDWARD S. JOSEPHSON, D. JANE TAYLOR, JOSEPH GREENBERG, AND AMAR P. RAY.
(Introduced by N. B. Eddy)

From the Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Md., and Malaria Institute of India, Delhi, India.

Berliner(1) observed that pamaquine (8-(4 - diethylamino - 1 - methylbutylamino)-6-methoxyquinoline), though markedly plasmodicidal *in vivo*, was relatively inactive when added to cultures of *Plasmodium falciparum in vitro*. Geiman(2) made essentially the same observation for the related 8-aminoquinolines, pentaquine and isopentaquine, using *in vitro* cultures of *P. knowlesi*. Berliner (1) also found that a solution of pamaquine, which had aged for several years, was highly active *in vitro*. Zubrod, Kennedy and Shannon(3) found that pamaquine was so extensively metabolized in the vertebrate body that extremely little of the drug was recovered in the excretory products of treated patients. Brodie and Udenfriend(4) and Hughes and Schmidt(5) have described metabolic intermediates of pamaquine and related 8-aminoquinolines, but none of these have been fully characterized and their antimalarial activities have not been determined. In an earlier report by three of us(6) it was shown that pamaquine, when added to *in vitro* cultures of *P. gallinaceum*, did not possess the marked antimalarial activity associated with the drug *in vivo*. In further trials (unpublished data) it was found that concentrates from the blood, tissues and droppings of chickens treated with pamaquine possessed antimalarial activity *in*

---

1. Berliner, R. W., personal communication.
2. Geiman, Q. M., Proceedings of the Fourth International Congresses on Tropical Medicine and Malaria, 1948, v1, 618.
3. Zubrod, C. G., Kennedy, T. J. and Shannon, J. A., *J. Clin. Inv.*, 1948, v27, 114.
4. Brodie, B. B., and Udenfriend, S., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 845.
5. Hughes, H. B., and Schmidt, L. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 581.
6. Greenberg, J., Josephson, E. S., and Taylor, D. J., *J. Inf. Dis.*, in press.

vitro which could not be accounted for on the basis of the pamaquine present in these concentrates. The foregoing suggested that the antiplasmodial and possibly the toxic, methemoglobin-forming properties of pamaquine are the result of metabolic transformation products of the drug. We, therefore, began our studies of the metabolites of pamaquine with the hope that we might be able to isolate a product or products which possessed antimalarial activity and, at the same time, be without toxic properties.

This report deals with the description of an intermediate obtained from chickens treated with pamaquine.

Methods. The product to be described has been obtained by essentially the same method from homogenized tissues (pooled blood, spleen, kidney, lung and pancreas) of treated chickens and from their droppings. However, since the amounts recovered from tissue homogenates were extremely minute, this report will be confined to the description of the product obtained from chicken droppings. Male and female New Hampshire Red chickens weighing 1500-2500 g were each given a single oral dose of pamaquine citrate (20 mg/kg body weight). Droppings were collected over an 18-hour period. Each 100 g of droppings were extracted with 600 ml of ethylene dichloride by shaking for 30 minutes. The organic phase, after separation of the solid residue by filtration, was washed twice for 15 minutes with approximately one-half volume of pH 8.8 buffer. After the second washing the final product was concentrated into about 1/100 volume of aqueous 0.1 N HCl. Partial purification was done with a 9 plate countercurrent distribution between ethylene dichloride and pH 7.6 buffer. Further purification and crystallization were impossible because of the small amounts of material available. The final extract was tested for the presence of pamaquine by the method of Brodie, Udenfriend and Taggart (8). Practically no measurable pamaquine was discovered in the chicken droppings. In fact, no material which would couple with diazotized sulfanilic acid was found in the crude ethylene dichloride extracts of the droppings of pamaquine-treated chickens. This would indicate that there was no pamaquine or ethylene dichloride soluble 8-aminoquinoline with an unsubstituted hydrogen in the 5 position present in the droppings. Ultraviolet absorption spectra were obtained, using the Beckman spectrophotometer Model DU. To test for the antimalarial activity of the degradation product *in vitro, P. gallinaceum* was used. Insufficient amounts were obtained to test the material *in vivo*. A method for the maintenance of *P. gallinaceum in vitro* and the methods employed for the assay of drug activity in such cultures have been described in detail(6,7). At the end of the 24-hour incubation period of the test material with *P. gallinaceum* the amount of methemoglobin present in the cultures was estimated by Wendel's adaptation(9) of the method of Evelyn and Malloy to the Coleman Junior Spectrophotometer, Model 6A. Additional changes in the procedure were necessitated by the fact that the chick erythrocyte in the cultures is resistant to hemolysis and contains a nucleus. Complete hemolysis with distilled water required one hour and the nuclear material and stroma were settled after centrifuga-

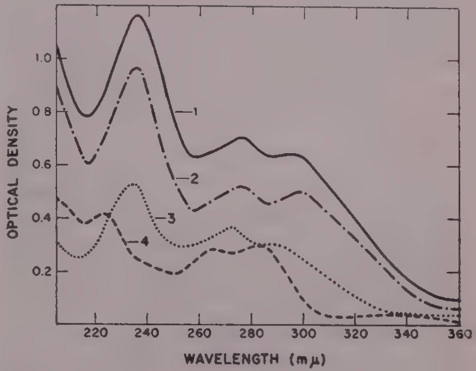


FIG. 1.

Absorption spectra of a metabolite of pamaquine from chickens (Curve 1), a product from the oxidative degradation of pamaquine by irradiation with ultraviolet (Curve 2), 8-amino-5,6-quinolinequinone (Curve 3), and pamaquine (Curve 4) in 0.1 N HCl.

---

7. Taylor, D. J., Greenberg, J., Josephson, E. S., and Ray, A. P., *J. Inf. Dis.* in press.

8. Brodie, B. B., Udenfriend, S., and Taggart, J. V., *J. Biol. Chem.*, 1947, v168, 327.

9. Wendel, W. B., personal communication.

tion for ten minutes at 3000 r.p.m.

Experimental. The ultraviolet absorption spectrum of the metabolite of pamaquine in 0.1 N HCl is shown in Fig. 1 (curve 1). Concentrates of the metabolite in 0.1 N HCl are orange-red. Dilute solutions of the metabolite in 0.1 N HCl are yellow. When the aqueous acid solution was frozen, the metabolite separated out as an orange-red oil. It was stable for at least 2 weeks when stored in the frozen state in aqueous acid solution at −18°C. The metabolite did not form a colored complex with diazotized sulfanilic acid, its absorption from wavelength 400 to 500 mμ being the same whether it was treated with sulfanilic acid or diazotized sulfanilic acid.

Estimations of the concentration of the metabolite in aqueous acid were based upon a comparison of its absorption of light at wavelength 420 mμ with that of pamaquine. This assumes that the molecular weight of the two compounds was roughly equal and that molecule for molecule they both absorb equally at 420 mμ. On this basis the metabolite was significantly active *in vitro* at concentrations of 625 μg/liter, while pamaquine was inactive at concentrations of less than 10,000 μg/liter (Table I). Furthermore, the metabolite produced significant methemoglobin at concentrations of 1250 μg/liter, while pamaquine produced none at concentrations below 20,000 μg/liter (Table I).

Discussion. It would be well to compare our metabolite with those obtained recently by several investigators studying the metabolic fate of 8-aminoquinolines. Brodie and Udenfriend(4) found 2 metabolites of pamaquine in the urine and blood of treated humans and dogs. One of these was stable and non-toxic as determined by its ability to lyse red cells and produce methemoglobin; the other was unstable and toxic by the same criteria. The ultraviolet absorption spectrum of their stable degradation product does not correspond to our metabolite, from which it also differs in that it does not produce methemoglobin *in vitro*. Since they have not been able to characterize fully their unstable, methemoglobin-inducing substance, comparison with our product is impossible. How-

TABLE I. *In vitro* Antimalarial and Methemoglobin-producing Properties of Pamaquine, a Metabolite of Pamaquine from Chickens, a Product from the Ultraviolet Irradiation of Pamaquine and 8-amino-5,6-quinolinequinone (DR 15,897).

	No. of parasites surviving 24-hr exposure*				Methemoglobin in cultures after 24 hr. % total hemoglobin†			
Conc. in culture (μg/l)	Pamaquine	Metabolite of pamaquine from chickens	Irradiation product from pamaquine	DR 15,897	Pamaquine	Metabolite of pamaquine from chickens	Irradiation product from pamaquine	DR 15,897
300	—	—	4×10^8	—	—	—	—	—
625	—	2.5×10^7	5×10^6	4×10^8	—	5.9	7.9	—
1250	—	5×10^6	1.25×10^6	2×10^8	—	19.4	6.9	—
2500	—	3.75×10^6	5×10^5	5×10^6	—	28.6	12.4	20.6
5000	2.5×10^8	1.25×10^5	5×10^4	5×10^4	—	42.9	35.2	29.0
10000	2.0×10^5	—	—	—	5.6	—	—	—
20000	5.0×10^3	—	—	—	6.6	—	—	—
40000	<1	—	—	—	22.4	—	—	—

* 5×10^8 parasites were initially present in each culture. Numbers of parasites in control cultures surviving 24 hr incubation ranged between 5×10^7 and 4.5×10^8. Differences less than 10-fold in numbers of viable parasites surviving treatment are not considered significant.

† In control cultures methemoglobin (% total hemoglobin) ranged between 3.8 and 7.4.

ever, it should be noted that our material is relatively stable in aqueous acid solutions.

Hughes and Schmidt(5) described degradation products of 8-aminoquinolines from plasma of monkeys, which differ among themselves in their solubility characteristics, but all of which form colored complexes with diazotized sulfanilic acid. Our degradation product obviously does not fit in their group of aqueous alkaline soluble, ethylene dichloride insoluble, compounds. Nor, does it fit in their group of ethylene dichloride soluble compounds which couple with diazotized sulfanilic acid. It should be mentioned that in our experience 8-aminoquinolines are degraded into a number of substances or complexes, so that it is possible for different investigators to select for characterization several metabolites whose properties are markedly different. Elderfield and Werble(10) obtained an 8-amino-5,6-quinolinequinone from the destructive irradiation of pamaquine with ultraviolet light, which they kindly sent us for testing. Their product corresponds quite closely to our metabolite in antimalarial activity and methemoglobin-forming properties *in vitro* (Table I). Their material also does not couple with diazotized sulfanilic acid and the ultraviolet absorption spectrum of their product and ours are almost identical (Fig. 1, curve 2). Finally, it should be mentioned that Drake and Pratt(11) have synthesized 8-amino-5,6-quinolinequinone (DR 15,897) as a possible degradation product of pentaquine. This material resembles our metabolite in its absorption characteristics over most of the ultraviolet range (Fig. 1, curve 3). However, it differs in its solubility characteristics, being much less soluble in aqueous acid solutions than our metabolite. DR 15,897 corresponds with our metabolite in *in vitro* antimalarial activity and methemoglobin-inducing properties from 2500 to 5000 μg/liter (Table I). Below this concentration the antimalarial activity of DR 15,897 decreased sharply, so that it was essentially inactive at 1250 μg/liter. Data on methemoglobin-inducing properties below 2500 μg/liter were not obtained. The properties of this synthetic quinolinequinone and the metabolite are similar enough to suggest a close structural relationship. Our metabolite is probably a quinolinequinone with a relatively undegraded 8-position sidechain as compared with DR 15,897. A difference in the structure of the sidechain could account for the discrepancies in solubility characteristics and absorption in the ultraviolet. If our product were a quinolinequinone it would substantiate the theory of Schönhöffer(12) which postulates that 6-methoxy-8-aminoquinolines are degraded through hydroxyquinolines to quinolinequinones.

It should be noted that while our product appears to be 16 times as active and toxic *in vitro* as pamaquine, the ratio of minimal antimalarial concentrations to minimal methemoglobin-forming concentrations is the same (1:4) for both compounds. It is possible that pamaquine is degraded in minute amounts in our culture vessels to yield active metabolites. However, the hope of obtaining an active metabolite which is non-toxic has not been realized in our preparation.

Summary. A metabolite of pamaquine hitherto undescribed has been obtained from the tissues and droppings of treated chickens. The *in vitro* antimalarial and methemoglobin-producing properties of this metabolite are approximately 16-fold those of pamaquine. There are striking similarities in ultraviolet absorption spectra and *in vitro* antimalarial and methemoglobin-inducing behavior of the metabolite and the two 8-amino-5,6-quinolinequinones studied.

---

10. Elderfield, R. E., and Werble, E., personal communication.

11. Drake, N. B., and Pratt, Y., *J. Am. Chem. Soc.* in press.

12. Schönhöffer, F., *Z. physiol. Chem.*, 1942, v274, 1.

Received February 23, 1951. P.S.E.B.M., 1951, v76.

Inhibition of Influenza Virus Hemagglutination by Purified Plasma Mucoproteins.* (18600)

C. S. Stulberg, Ruth Schapira, Abner R. Robinson, Daniel H. Basinski, and Hugo A. Freund.

From the Research Laboratory, Children's Fund of Michigan, Detroit, Mich.

The adsorption of influenza virus to red cells, followed by red cell receptor destruction and subsequent elution of virus, have been suggestive of an enzymatic reaction(1). The probable substrates for such enzymatic activity have been shown to be mucopolysaccharides or mucoproteins present in red cell receptors(2,3). These mucoid substances, also found in plasma(4), in blood group substances(2), and in other biological materials (2,5), appear to act in competition with red cell receptors for virus in the virus hemagglutination reaction(6). The chemical nature of these mucoids remains obscure in spite of several investigations concerned with their isolation and identification(7-9). Recently, however, Winzler *et al.*(10) have isolated and tentatively characterized plasma mucoproteins with low isoelectric points, both chemically and electrophoretically.

This report describes the inhibition of influenza virus hemagglutination by an electrophoretically homogeneous mucoprotein.

Isolation of mucoprotein. The method of Winzler *et al.*(10), with slight modifications, was used for the preparation of mucoprotein from out-dated, blood-bank plasma. The plasma proteins were first removed by precipitation with 1.8 M perchloric acid. The filtrate then was dialyzed and adjusted to pH 4. Ammonium sulfate was added to the filtrate and the mucoprotein began to precipitate when the concentration was approximately 2/3 that of saturation. Infusorial earth (Johns-Manville hyflo super cel) was added to make a concentration of approximately 40 g per liter of mixture. After stirring for a few minutes, the mucoprotein was completely adsorbed on the infusorial earth. After filtration the mucoprotein was eluted with 5 100 ml portions of distilled water and the solution was saturated with ammonium sulfate, and refiltered. The re-precipitated mucoprotein was dissolved in a small amount of water and dialyzed. The salt-free mucoprotein was dried by lyophilization. The average yield was slightly more than 200 mg per liter of plasma. Two preparations, designated as A and B, were obtained in this manner; another preparation, C, was obtained by omitting the adsorption and drying the final product by evaporation at room temperature. Data from analyses of mucoprotein preparation A are given in Table I, and are consistent with unpublished analyses of several preparations. The total nitrogen content is in agreement with values reported by Weimer, *et al.* (11), but the tyrosine and carbohydrate values are somewhat lower than reported by those authors.

Electrophoretic characterization of muco-

---

* This report is a part of one phase of the arthritic research program initiated and directed by Hugo A. Freund, and supported, in part, by funds contributed by the National Foundation of Rochester, Michigan and the Michigan Chapter, Arthritis and Rheumatism Foundation. Blood samples for this work were obtained from the Children's Hospital of Michigan.

1. Hirst, G. K., *J. Exp. Med.*, 1942, v76, 195.
2. Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Aust. J. Exp. Biol. and Med. Sci.*, 1948, v26, 403.
3. Hirst, G. K., *J. Exp. Med.*, 1948, v87, 301.
4. Hirst, G. K., *J. Exp. Med.*, 1949, v89, 223.
5. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, v179, 1275.
6. Hirst, G. K., *J. Exp. Med.*, 1948, v87, 315.
7. McCrea, J. F., *Aust. J. Exp. Biol. and Med. Sci.*, 1948, v26, 355.
8. De Burgh, P. M., Pen-Chung, Y., Howe, C., and Bovarnick, M., *J. Exp. Med.*, 1948, v87, 1.
9. Howe, C., *J. Immunol.*, 1951, v66, 9.
10. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.
11. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *Fed. Proc.*, 1950, v9, 244.

TABLE I. Properties of Purified Plasma Mucoprotein (Preparation A).

Mobility, cm²/sec./volt*	4.3×10^{-5}
Kjeldahl N %	10.9
Tyrosine %	1.7
Carbohydrate %	9.6-12.2
Inhibition titer†	4096-8192
μg mucoprotein at inhibition endpoint	0.3-0.15

* Acetate buffer pH 4.5, ionic strength 0.1.

† Reciprocal of dilution starting with 1.25 mg mucoprotein.

TABLE II. Inhibitory Effects of Purified Mucoprotein Preparations on Hemagglutination by Heated and Unheated Influenza Virus.

Mucoprotein (5 mg/ml)	Inhibition titer	
	Unheated virus	Heated virus
Preparation A	1024	4096- 8192
,, B	1024-4096	4096- 8192
,, C	variable	4096-16384

protein. The 3 preparations of isolated mucoprotein were studied electrophoretically at pH 4.5 as described by Mehl *et al.*(12), using acetate buffer, pH 4.5, ionic strength 0.1. The electrophoretic determinations were made with the Longsworth modification of the Tiselius apparatus. Fig. 1 shows the single component which appeared in each preparation and migrated toward the anode with a mobility of 4.3 x 10^{-5} cm²/sec/volt.

Effects of mucoproteins on virus hemagglutination. The PR8 strain of influenza A virus was used in the hemagglutination tests. Pooled allantoic fluids, harvested 48 hours after inoculation of 10-day-old chick embryos with a 10^{-3} dilution of seed virus, were frozen or were refrigerated at 4°C. Aliquots of the same pools were heated at 56°C for 30 minutes. In a modified Salk pattern test, agglutination occurred consistently at a dilution of 1:5120 with the unheated virus, and of 1:2560 with the heated virus. For the virus hemagglutination-inhibition tests, the lyophilized mucoprotein samples were carefully weighed to an accuracy of 0.1 mg and diluted with 0.85% NaCl solution until they contained the desired concentration of mucoprotein. This mucoprotein solution then was diluted in serial two-fold dilutions in 0.25 ml volumes of physiological saline, and to each was added an equal volume of diluent containing 5 hemagglutination units of heated or unheated virus. After the mixture was incubated at room temperature for one hour, 0.5 ml of a 0.5% suspension of chicken red cells was added. The patterns of the sedimented cells were observed after incubation at 4°C, when agglutination of the cells was complete. The inhibition titer was arbitrarily read as the reciprocal of the highest dilution of mucoprotein which completely inhibited hemagglutination. The results of the inhibition tests are shown in Table II. It appears that all 3 mucoprotein preparations possess the capacity to inhibit hemagglutination by heat-inactivated virus. The inhibition titers of preparations A and B were approximately the same, which might be considered further evidence of the homogeneity of the purified product. With heat-inactivated virus the hemagglutination inhibition titers of preparations A and B ranged from 4096 to 8192, the minimal inhibition concentrations being approximately 0.15 to 0.3 μg of mucoprotein. Preparation C exhibited somewhat more variable inhibition titers, 4096 to 16,384. The mucoprotein preparations inhibited unheated, or active, virus hemagglutination at a lower and often variable dilution. The results with unheated virus paralleled the observations of previous investigators, who demonstrated that heat-inactivated virus does not destroy virus inhibitor, whereas unheated virus affects inhibitors to an extent which depends upon the experimental conditions employed.

Mucoproteins and hemagglutination-inhibition properties of heated serum. Both McCrea(7) and Hirst(4) have demonstrated the ability of virus hemagglutination inhibitor in plasma to withstand a temperature of 100°C. Both studied heat coagulation methods in attempts to identify this inhibitor as seromucoid. It was pertinent, therefore, to study the electrophoretic characterization at pH 4.5 and the agglutination-inhibition properties of heated human sera. Serum samples diluted approximately 1 to 3 with acetate buffer, pH 4.5, were heated at 100°C for 3 minutes. The

---

12. Mehl, J. W., Humphrey, J., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 106.

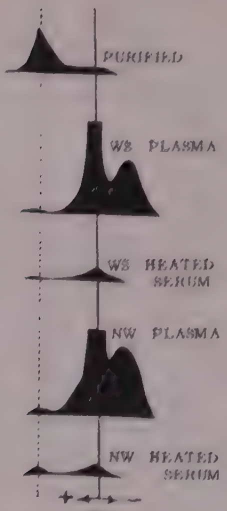


FIG. 1.

Electrophoresis patterns of preparations in acetate buffer, pH 4.5, ionic strength 0.1, showing the mucoprotein component (broken line) in purified solution, in plasma, and in heated serum.

uncoagulated supernate was prepared for electrophoretic examination according to the method of Mehl *et al.*(13). For the hemagglutination-inhibition tests, aliquots of the dialyzed samples used for electrophoresis were brought to pH 6.5-6.9 with NaOH and corresponding serial two-fold dilutions prepared with 0.85% saline.

Electrophoretic analyses of plasma and heated serum from 2 individuals representative of those with relatively low (WS) and high (NW) mucoprotein concentrations† are shown in Fig. 1. In the heat-treated specimens, a component appeared which had a mobility of 4.1-4.3 x 10^{-5} cm^2/sec/volt and was therefore considered to be a mucoprotein. These mobilities were in close agreement with that of the single component observed in the purified preparations and with the mobilities of similar components in unheated plasma from the same individuals. The relative mucoprotein concentrations of the plasma and heat-treated sera of WS and NW, together with their corresponding hemagglutination-inhibition titers, are shown in Table III. When more mucoprotein was present, higher inhibition titers were obtained, which indicates a relationship of the inhibitor to mucoprotein; however, these inhibition titers with unpurified substances may only indicate relative amounts of mucoprotein, for the differences in titers are not proportional to the differences in mucoprotein concentrations. From the electrophoretic patterns of the heated sera, it was evident that in addition to the mucoprotein, another component was present which had little mobility at pH 4.5, and this component may have affected the activity of the mucoprotein as an inhibitor.

Summary. Mucoprotein was isolated from human plasma by the perchloric acid filtrate method. This isolated mucoprotein was found to be electrophoretically homogeneous, and consistently inhibited hemagglutination by influenza virus in dilutions which contained 0.15 to 0.3 μg of mucoprotein. Hemagglutination inhibition with heated serum also indicated that the inhibitory substance was a mucoprotein.

TABLE III. Mucoprotein Concentrations of Human Plasma and Relative Mucoprotein Concentrations and Inhibition Titers of Heat-treated Human Sera.

Blood source	Plasma mucoprotein, mg/100 ml	Heated serum mucoprotein, mg/100 ml	Inhibition titer with heated virus
WS	77	84	16-32
NW	205	155	256-1024

Received February 26, 1951. P.S.E.B.M., 1951, v76

---

13. Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 110.

† In a study of plasma mucoproteins in rheumatoid arthritis, in this laboratory(14) the plasma of arthritic patients has been found to contain much higher concentrations of mucoprotein than that of normal individuals. In the present investigation, WS was normal while NW had rheumatoid arthritis.

14. Unpublished data.

Increase in Circulating Red Cell Volume of Normal and Hypophysectomized Rats after Treatment with ACTH.* (18601)

JOSEPH F. GARCIA, DONALD C. VAN DYKE, REX L. HUFF, PAUL J. ELMLINGER,† AND JOHN M. ODA. (Introduced by J. H. Lawrence)

From the Division of Medical Physics, and the Institute of Experimental Biology, University of California, Berkeley.

The presence of low hemoglobin concentrations in hypophysectomized animals was first noted in hypophysectomized dogs by Aschner (1). That this low concentration actually reflects a decrease in red blood cell volume per unit body weight has been shown by the P^{32} tagged cell method(2). The repair of this anemia has been the subject of many papers(4,8,11,12). Some authors(8) conclude that the alterations in red cell concentration following hypophysectomy are due to "general metabolic changes" rather than the absence of a specific hemopoietic hormone. On the other hand, a specific hemopoietic hormone has been postulated as existing in the hypophysis(5,6,9).

The general subjects of red blood cell production, destruction and volume in relation to endocrine secretions have received little attention because of lack of methods for their determination. However, concentrations of red cells and hemoglobin in large vessels and changes in these concentrations subsequent to or concomitantly with clinical and experimental endocrine changes have been studied extensively. The points of immediate interest are total red cell volumes, marrow volumes, and in particular, the manner in which these volumes change. This report concerns only the changes in total circulating red cell volume per unit mass of tissue in normal rats and hypophysectomized rats with and without treatment with ACTH.

Methods. Ninety-day-old female Long-Evans rats were studied in 4 groups of 10 animals each: Group (1) hypophysectomized control, group (2) hypophysectomized receiving 1 mg ACTH daily, group (3) normal control, group (4) normal receiving 1 mg ACTH daily. The animals were hypophysectomized by the parapharyngeal approach on the first day of the experiment. The ACTH was given in ½ mg intraperitoneal doses twice daily for 34 days to half the members and for 116 days to the rest of the members of the groups receiving the hormone. The total red cell volumes were determined with P^{32} tagged cells using the method of Hevesy (7) as modified by Berlin *et al.*(3). Each animal was anesthetized with ether and the tagged cells were injected into the saphenous vein and allowed to mix for 5 minutes, at which time the abdomen was opened and as much blood as possible was drawn from the inferior vena cava into a heparinized syringe. The P^{32} was assayed on an aliquot of this blood with the aid of an end window Geiger-Muller counter. Hematocrits were done with calibrated capillary tubing. The pituitary

---

* This work was supported by grants from the Atomic Energy Commission and the U. S. Public Health Service, Grant C-1098.

† Post Doctorate Research Fellow of the Atomic Energy Commission.

1. Aschner, B., *Pflüg. Arch. f. d. ges. Physiol.*, 1912, v146, 1.

2. Berlin, N. I., Van Dyke, D. C., Siri, W. E., and Williams, C., *Endocrinology*, 1950, v47, 429.

3. Berlin, N. I., Huff, R. L., Van Dyke, D. C., and Hennessy, T. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 176.

4. Crafts, R. C., *Am. J. Anat.*, 1946, v79, 267.

5. Flaks, J., Himmel, I., and Zlotnik, A., *Presse Med.*, 1938, v46, 1506.

6. Flaks, J., Himmel, I., and Zlotnik, A., *Presse Med.*, 1937, v45, 1261.

7. Hevesy, G., and Zerahn, K., *Acta physiol. Scand.*, 1942, v4, 376.

8. Meyer, O. O., Thewlis, E. W., and Rusch, H. P., *Endocrinology*, 1940, v27, 932.

9. Moehlig, R. C., and Bates, G. S., *Arch. Int. Med.*, 1933, v51, 207.

10. Sundstroem, E. S., and Michaels, G., *Memoirs of the University of California*, 1942, v12, 42.

11. Vollmer, E. P., Gordon, A. S., and Charipper, H. A., *Endocrinology*, 1942, v31, 619.

12. Vollmer, E. P., and Gordon, A. S., *Endocrinology*, 1941, v29, 828.

TABLE I. Total Circulating Red Cell Volume in Normal and Hypophysectomized Rats Treated with Adrenocorticotropic Hormone.

	Exp. terminated after 34 days				Experiment terminated after 116 days				
	Rat No.	Terminal body wt, g	Hct., %	Red cell vol., cc/100 g body wt	Rat No.	Terminal body wt, g	Adrenal wt (2 adrenals) mg	Hct., %	Red cell vol., cc/100 g body wt
Hypophysectomized controls	1	182	41.4	1.71	21	died	—	—	—
	2	155	37.5	1.44	22	died	—	—	—
	3	148	35.0	1.44	23	152	10	29.5	1.64
	4	178	27.2	1.31	24	153	13	28.9	1.44
	5	153	32.8	1.64	25	died	—	—	—
	Avg		34.8	1.51	Avg		11.5	29.2	1.54
Hypophysectomized receiving .5 mg ACTH twice daily	6	190	39.0	1.87	26	165	62	46.9	2.37
	7	184	38.6	2.21	27	128	56	43.6	2.54
	8	132	35.5	2.38	28	164	56	41.6	2.41
	9	146	39.2	2.15	29	died	—	—	—
	10	153	40.3	2.34	30	173	54	39.4	2.21
	Avg		38.5	2.19	Avg		57	42.9	2.38
Normal controls	11	235	46.3	2.42	31	290	52	47.6	2.25
	12	222	45.7	2.80	32	277	42	48.3	2.35
	13	210	44.2	2.76	33	242	62	49.0	2.66
	14	187	43.6	2.73	34	247	61	45.9	2.33
	15	204	40.7	2.50	35	253	67	45.5	2.32
	Avg		44.1	2.62	Avg		57	47.3	2.38
Normal rats receiving .5 mg ACTH twice daily	16	236	47.0	2.58	36	225	137	55.2	3.27
	17	225	40.0	2.43	37	Accidental blood loss			
	18	224	50.3	3.01	38	255	114	49.2	2.93
	19	213	45.3	2.95	39	278	106	55.2	3.24
	20	190	45.3	2.35	40	233	106	48.5	2.96
	Avg		45.6	2.66	Avg		116	52.0	3.10

site was examined under a binocular dissecting microscope to check the completeness of hypophysectomy.

Results. The data are presented in Table I. The red cell volumes are given in terms of cubic centimeters of packed red cells per hundred grams of body weight. In both of the hypophysectomized control groups the total red cell volumes were considerably below the values obtained for the normal animals, and in agreement with the total red cell volumes in hypophysectomized rats as shown by Berlin *et al.*(2). However, the group of hypophysectomized animals receiving 1 mg ACTH daily for 34 days showed a significant increase in total red cell volume as compared to the untreated hypophysectomized controls. The average total red cell volume for these ACTH treated hypophysectomized animals was 2.19 cc/100 g body weight as compared to 1.51 cc/100 g body weight for the untreated hypophysectomized animals sacrificed at this same time period. The normal animals receiving 1 mg ACTH daily for 34 days showed no significant increase in their total red cell volume when compared with their controls. The average red cell volume for the ACTH treated normals was 2.66 cc/100 g body weight and for the untreated normal controls was 2.62 cc/100 g body weight.

The total red cell volumes in hypophysectomized animals after 116 days of treatment with 1 mg ACTH daily increased greatly (2.38 cc/100 g body weight) over the value for total red cell volume in hypophysectomized controls 116 days after hypophysectomy (1.54 cc/100 g body weight), and in fact were brought to within the range of the total red cell volumes (2.38 cc/100 g body weight) in normal animals of the same age.

Although no significant change in total red cell volume was seen in normal animals after 34 days of treatment with ACTH, there was a significant polycythemia produced after 116 days of ACTH treatment. An average total red cell volume of 3.10 cc/100 g body weight was produced in these animals as compared to the average of 2.38 cc/100 g body weight for normal controls at this same age.

The average total red cell volumes for the 2 groups of normal controls differ slightly; however, when significance tests were applied, this difference was shown not to be significant.

The adrenal weights were measured in the animals autopsied at 206 days of age. As can be seen from the table, these weights are directly related to the red cell volume data. The untreated hypophysectomized rats had an average adrenal weight of 11.5 mg (2 adrenals). In the hypophysectomized rats treated with ACTH the adrenal weights gave the same average (57 mg) as did the normal controls. The normal rats treated with ACTH showed an increase in adrenal weight with an average of 116 mg.

These data are interesting in view of the fact that Sundstroem(10) found, along with the well known polycythemia, greatly increased adrenal weights present in rats placed in an atmosphere of reduced oxygen tension.

Conclusions. 1. Administration of ACTH prevents the decrease in the total circulating red cell volume which is normally found in hypophysectomized rats. 2. Administration of ACTH for 116 days to normal rats elevates the total circulating red cell volume to approximately 1.3 times that of the normal untreated controls.

ACTH was supplied through the courtesy of Professor Choh Hao Li, Department of Biochemistry, University of California, Berkeley.

Received February 26, 1951. P.S.E.B.M., 1951, v76.

P^{32} Localization in the Intestine. (18602)

MARGARET W. HOLT AND SHIELDS WARREN.

From the Cancer Research Institute, New England Deaconess Hospital and Laboratory of Pathology, Harvard Cancer Commission, Boston, Mass.

Radioautographic and histochemical observations have been made on sections of frozen-dried rat and mouse intestine containing P^{32}. The tissue sections were prepared and mounted and radioautographs were made using procedures reported previously(1,2). These procedures are designed toward qualitative and quantitative preservation and retention at the original sites of tissue constituents. The radioautographs of rat and mouse duodenum and small intestine have shown a more striking concentration and localization pattern with P^{32} than has been observed for other soft tissues. This localization occurs in the glands of the mucosa with the greater concentration at the more basal groups of cells (Fig. 1).

Studies of the solubility of localized P^{32} in frozen-dried sections of rat intestine have been reported(3). The localized P^{32} exhibits the same general solubilities as the background P^{32} in other regions of the tissue, indicating a similar chemical form. The P^{32} is not in phospholipid form as it does not dissolve in organic solvents; other solubility tests have indicated the presence of free phosphate ions and at least a partial protein linkage. The use of formalin or Bouin's solution as a fixative results in a radioautograph showing the same general type of localization pattern, but of diminished intensity as compared to the results with frozen dried tissues. This would be expected on the basis of solubility data.

Observations made at varying time intervals from isotope administration to killing the animal have shown that the typical localization pattern is present in the intestine at 30

---

1. Holt, M. W., Cowing, R. F., and Warren, S., *Science*, 1949, v110, 328.

2. Holt, M. W., and Warren, S., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 545.

3. Holt, M. W., and Warren, S., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 4,

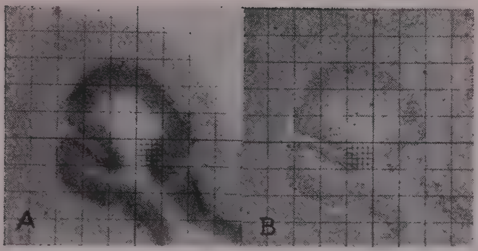


FIG. 1a. Radioautograph of rat intestine prepared from an approx. 2 μ tissue section. The tissue was mounted on a cover glass which was clamped so that it faced the emulsion of a contrast lantern slide. Direct contact of the tissue with the emulsion was prevented by placing "spacers" which consisted of strips of tantalum foil approx. 5 μ in thickness around the tissue section. The tissue and emulsion surfaces were therefore separated by a distance of approx. 3 μ. Isopentane at —160 to —195°C, paraffin, and xylol were the only reagents which came into contact with the tissue previous to the radioautographic exposure. 237 μc of carrier free P^{32} were administered subcutaneously to a 65 g rat for a 3½ hr time interval. A 16-day exposure was used which was started 9 days following the isotope injection.

FIG. 1b. Feulgen stained tissue section which corresponds to the radioautograph shown in *a*. The dark areas which show the most intense reaction are in the basal mucosal layers, the positive reaction being located in the nuclei of the cells.

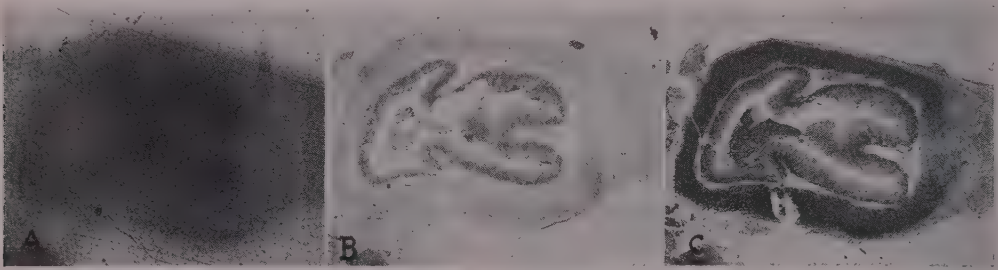


FIG. 2a. Radioautograph of frozen-dried rat intestine. The tissue, in the process of preparation, was exposed only to Isopentane at —160 to —195°C, paraffin, and xylol. 500 μc were administered subcutaneously to a 120 g rat for a 4-hr time interval. A 9-day exposure to contrast lantern slide was started 10 days following isotope injection.

FIG. 2b. A routine hematoxylin and eosin stain of a tissue section which corresponds to the radioautograph shown in *a*.

FIG. 2c. Alkaline phosphatase (Gomori) reaction on a tissue section which corresponds to those shown in *a* and *b*. The sites of high phosphatase concentration are shown as darkened areas. Note these areas do not correspond in orientation to those of P^{32} localization shown in *a*.

minutes, 2, 4, 8, 24, and 48 hours. Some of the rat and mouse intestine sections were stained with the Feulgen reagent following radioautographic exposure. The pattern of localization of P^{32} in the radioautograph was found to correspond exactly with the pattern of localization of desoxyribose nucleic acid in the Feulgen stained sections (Fig. 1a, b). We believe that this evidence identifies the P^{32} with that which is being incorporated into newly formed desoxyribose nucleic acid molecules in the rapidly dividing mucosal cells. However, from a quantitative standpoint, chemical analyses(4) have shown that the P^{32} incorporated into newly formed nucleic acid is only a minor fraction of the total intestinal P^{32} uptake. The radioautographs show that the major fraction of the P^{32} present in the intestine is distributed in the regions of high nucleic acid content. This suggests that localization of P^{32} in other chemical form (probably inorganic) occurs previous to or associated with the nucleic acid synthesis.

Other histochemical tests, on the other hand, have shown that the P^{32} localization does not correspond in position with the sites of high phosphatase concentration (Fig. 2a, b, c); and therefore that reactions involving this enzyme are not involved in the functional concentration of P^{32}.

This observed high local concentration of P^{32} associated with the high rate of nucleic acid turnover in the intestine is of particular importance in relation to the administration of P^{32} during various types of treatment and diagnostic procedures (for polycythemia, leu-

---

4. Lawrence, John H., and Hamilton, J. G., *Advances in Biological and Medical Physics*, New York, Academic Press Inc., 1948, v1, 430.

kemia, tumors, etc.), and should be considered as a potential radiation hazard in these procedures.

This work was done under A.E.C. Contract No. At(30-1)-901. The P^{32} used was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn. We wish to acknowledge the technical assistance of Misses Alice A. Williams and Elizabeth Stone.

Received February 26, 1951. P.S.E.B.M., 1951, v76.

Irradiation Produced Rise in Blood Radioiodine Concentration Following Ingested Therapeutic Dose for Metastatic Thyroid Carcinoma.* (18603)

S. M. Seidlin, A. Aaron Yalow, and Edward Siegel. (Introduced by L. Leiter)

From the Medical Physics Research Laboratory, Montefiore Hospital, New York.

In the study of physiological and pathological processes using radioactive substances, a clear differentiation must be made between the effects of tracer and therapeutic doses. While tracer doses have only a negligibly destructive effect upon living tissue, in therapeutic doses one deliberately administers a sufficient quantity of the isotope to produce tissue damage in selected areas.

In this paper we are concerned with the delayed rise in the blood radioiodine, usually occurring 4 to 8 days after ingestion of a therapeutic dose, and which we attribute to irradiation damage to the thyroid gland or thyroid tumor tissue. This rise is apart from the initial rise due to absorption of the isotope into the blood stream and a subsequent increase from 1 to 3 days later found in tracer studies by Pochin(1,2) and other investigators(3-5) and which they ascribe to the release of labeled thyroxine from the thyroid gland in the course of iodine metabolism of euthyroid and hyperthyroid individuals. McConahey, Keating and Power(6) have studied the effect of tracer and therapeutic doses of radioiodine for periods from 1 to 7 days. There is no reference in their paper to the rise in blood I* concentration reported here. They state that "one may be justified in assuming that the results (with therapeutic doses) observed correspond at least qualitatively to physiologic conditions." However, Marinelli, Trunnell and their associates(7,8) have occasionally observed transient hyperthyroidism with a rise in protein bound plasma iodine after administration of therapeutic doses of I^{131} to metastatic thyroid carcinoma patients. Marinelli and Hill(9) attribute this to "the release of thyroid hormone by functional cancer tissue under irradiation." This explanation is supported by the study on rats by Feller *et al.*(10), who have reported a drop in the stable iodine content of the thyroid and a rise in plasma protein bound iodine following intraperitoneal injection of massive I^{131} doses.

As was recently reported by one of us,† we have determined the pattern of radioiodine

---

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service and a grant from the G. N. Stewart Memorial Fund of the Rogoff Foundation.

1. Myant, N. B., and Pochin, E. E., *Proc. Roy. Soc. Med.,* 1949, v42, 959.

2. Pochin, E. E., *Lancet,* 1950, v259, 41 and 83.

3. Potts, A. M., Shipley, R. A., Storaacsli, J. P., and Friedell, H. L., *J. Lab. and Clin. Med.,* 1949, v34, 1520.

4. Rollman, H., and Petit, D. W., Proc. Soc. Exp. Biol. and Med., 1949, v72, 474.

5. Freedberg, A. S., Ureles, A., and Hertz, S., Proc. Soc. Exp. Biol. and Med., 1949, v70, 679.

6. McConahey, W. M., Keating, F. R., and Power, M. H., *J. Clin. Invest.,* 1949, v28, 191.

7. Marinelli, L. D., Trunnell, J. B., Hill, R. D., and Foote, F. W., *Radiology,* 1948, v51, 553.

8. Trunnell, J. B., Marinelli, L. D., Duffy, B. J., Hill, R. D., Peacock, W., and Rawson, R. W., *J. Clin. Endocrinol.,* 1949, v9, 1138.

9. Marinelli, L. D., and Hill, R. D., *Radiology,* 1950, v55, 494.

10. Feller, D. D., Chaikoff, I. L., Taurog, A., and Jones, H. B., *Endocrinol.,* 1949, v45, 464.

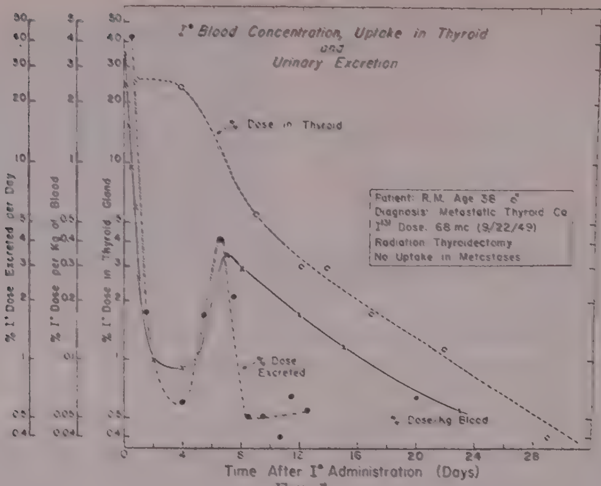


FIG. 1.

Demonstration of irradiation produced rise (IPR) in blood radioiodine concentration for patient receiving thyroidectomizing I^{131} dose. Note rise in blood I* concentration and urinary excretion occurring simultaneously with the sharp drop in radioiodine content of the thyroid gland.

concentration in the blood of patients with metastatic thyroid carcinoma who received large doses (50-200 mc) of radioiodine[‡] during radiation thyroidectomy, during irradiation of metastatic thyroid carcinoma lesions in the course of radioiodine therapy, and after the cessation of I* uptake in both the non-neoplastic thyroid gland and the metastases. Weighed blood samples were taken at intervals of decreasing frequency after the oral administration of radioiodine, and their activity was determined by gamma ray counting. The per cent dose present per kilogram of blood was obtained by comparing the activity of the sample with that of an appropriate aliquot of the administered dose.

In the treatment of metastatic thyroid carcinoma, a thyroidectomy, surgical or radiation, is performed in order to induce or increase the function of the metastases, and hence their ability to localize radioiodine selectively (11). When the thyroidectomy is to be accomplished by internal irradiation, the patient is given a large dose of radioiodine. As the radioiodine is absorbed from the stomach, the blood I* concentration rises within a few hours to a maximum value. We find that a sharp drop in the radioiodine content of the thyroid gland occurs 4 to 8 days following ingestion of the isotope. A simultaneous rise in the blood I* concentration and in the urinary radioiodine excretion is observed. An illustrative case is shown in Fig. 1.

For reasons presented below we designate this secondary rise as the *irradiation produced rise* (IPR). Its magnitude is taken to be the ratio of the maximum observed blood I* concentration (% dose/kg) to the concentration immediately preceding the rise. Rises of less than 1.10 were not considered significant. The IPR's observed in this series have ranged from 1.12 to 3.90 (Table I).

High I* concentration in tissue will result in an intense local irradiation and hence considerable radiation trauma. We attribute the rise in blood and urinary I* concentration to the release of radioiodine from the disintegrating irradiated thyroid tissue. It is, therefore, not surprising that, in general, the IPR increases with the quantity of radioiodine retained by the thyroid. In our opinion, the

---

[†] S. M. Seidlin, Sixth Intern. Cong. of Radiology, July 23-29, 1950, London, England.

[‡] All radioiodine administered since Sept., 1946 has been I^{131}, supplied on allocation from the Isotopes Division, Atomic Energy Commission.

11. Seidlin, S. M., Oshry, E., and Yalow, A. A., *J. Clin. Endocrinol.*, 1948, v8, 423.

TABLE I. Incidence of Secondary Rise in Blood I* Concentration (IPR) in Patients Receiving Therapeutic Doses of Radioiodine.

Group	Functional status of thyroid gland and metastases	No. of patients	No. of doses	Doses exhibiting IPR* No.	%	Avg IPR
A	Radiation thyroidectomy†	7	7	7	100	2.09
B	Low thyroid uptake‡ No demonstrable uptake in metastases	2	2	1	50	1.25
C	No demonstrable uptake in thyroid Uptake in metastases	13	33	8	24	1.35
D	No demonstrable uptake in thyroid No demonstrable uptake in metastases	4	4	0	0	—

* Only IPR $\geq$ 1.10 considered significant.

† Radioiodine uptake in thyroid $\geq$ 10% administered dose.

‡ Definite but slight I* uptake in thyroid, $<$ 10% of administered dose.

rise in the blood iodine concentration observed after administration of tracer doses of radioiodine to normals plays an insignificant role in the above-described phenomenon. We have also observed this IPR in totally thyroidectomized patients with functioning thyroid carcinoma metastases (Curve B.N., Fig. 2). As therapy proceeds, the quantity and the viability of functioning tissue decreases and along with it the magnitude of the IPR. The latter finally vanishes with the disappearance of the residual functioning tumor tissue (Curve S.W., Fig. 2). At this stage of therapy, the blood radioiodine concentration rapidly falls to very low values and there is no secondary rise.

In our series, the blood of 21 patients was studied for 5 or more days following administration of 46 therapeutic doses of radioiodine. Table I shows the incidence of secondary rises in blood I* concentration. A definite correlation is shown between the frequency and magnitude of the IPR and the functional level of thyroid tissue, neoplastic or non-neoplastic, present in the body. Since we have not found a secondary rise in blood I* concentration in the absence of functioning thyroid tissue, the occurrence of such a rise in a totally thyroidectomized patient might be considered evidence of viable functioning thyroid carcinoma metastases. It is apparent that for the usual cases, where there is Geiger counter or radioautographic evidence of uptake in the metastases, the IPR is merely an additional diagnostic aid. Similar methods for detection of metastases in other types of tumors might be developed should radioactive substances with specific affinity for these tumors be discovered.

Summary. Four to 8 days after ingestion of therapeutic doses of radioiodine (50-200 mc) by patients with metastatic thyroid carcinoma, a rise occurs in blood radioiodine concentration which we attribute to irradiation damage to the thyroid gland or thyroid

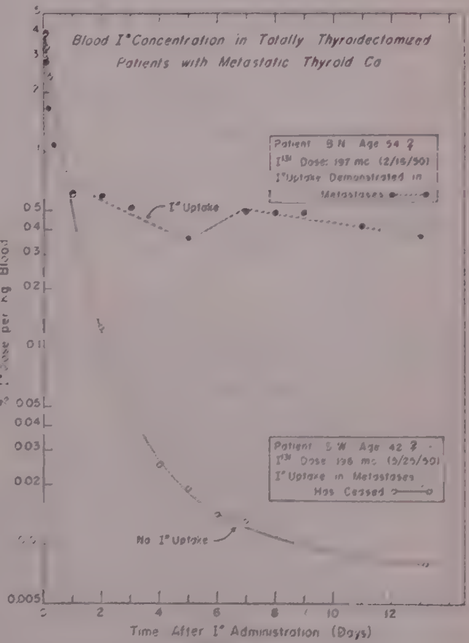


FIG. 2.

Dependence of IPR and I* blood level on function of thyroid metastases. The higher level and the IPR are observed in the patient (B.N.) having radioiodine uptake in metastases.

tumor tissue. This increase we have termed the irradiation produced rise. In a series of patients the magnitude of the IPR in general has been found to increase during radiation thyroidectomy with the quantity of radioiodine retained by the thyroid. In a given patient the magnitude and incidence of the IPR decrease with successive doses as functioning thyroid tissue is progressively destroyed. Finally, when there is no longer any functioning tissue, as demonstrated by absence of I* uptake, the blood radioiodine concentration rapidly falls to very low levels and there is no IPR.

The blood of 21 patients, in various stages of therapy, was studied. For all of 7 radiation thyroidectomy doses, significant IPR's were found; in thyroidectomized patients with functioning metastases, IPR's were demonstrated in 8 of 33 doses. However, no such rise in blood iodine concentration was observed following four doses to thyroidectomized patients lacking demonstrable I* uptake. Since no such rise is found in the absence of functioning thyroid tissue, either neoplastic or non-neoplastic, the occurrence of an IPR in a thyroidectomized patient may, therefore, be considered evidence of the existence of viable functioning thyroid carcinoma metastases.

Received January 22, 1951. P.S.E.B.M., 1951, v76.

Changes with Age in Amino Acid Composition of Arterial Elastin.*

(18604)

A. I. LANSING, EUGENE ROBERTS, G. B. RAMASARMA,[†] THEODORE B. ROSENTHAL, AND MORRIS ALEX.

From the Department of Anatomy and the Wernse Laboratory of Cancer Research, Washington University School of Medicine, St. Louis.

During a study of human arteriosclerosis it became evident that changes in the media of the aorta preceded the formation of intimal plaques(1,2). In particular, the elastic tissue of the tunica media, free of minerals in youth, became heavily calcified in adult and aging individuals.

Elasto-calcinosis, as the process of calcification of arterial elastic tissue has been termed recently(1), is accompanied by a number of alterations in the morphologic appearance and tinctorial reactions of elastic fibers. In the light of these differences, we have initiated a program to compare the chemical properties of young and old elastic fibers. The changes with age in the amino acid composition of human arterial elastin are now being reported.

Experimental. Analytical procedures. Elastin was prepared from human aortas (aortic elastin) and pulmonary arteries (pulmonary elastin) and calcium determinations were made on ashed samples by methods which have been described previously(3). The nitrogen contents were determined and the amino acid distributions were made by microbiological and paper chromatographic methods, the details of which have also been given previously(4). Several phosphate determinations on the ash from elastin gave a molar Ca:P ratio of approximately 3:2. For purposes of calculation it was, therefore, assumed

---

[*] Aided by grants from the National Cancer Institute, the National Heart Institute of the National Institutes of Health, U. S. P. H. Service; from the Charles F. Kettering Foundation; and from the American Heart Association.

[†] Eli Lilly Fellow, 1950. Permanent address, Raptakos Brett and Co., Bombay, India.

1. Lansing, A. I., Alex, M., and Rosenthal, T. B., *J. Gerontol.*, 1950, v5, 314.

2. Lansing, A. I., Alex, M., and Rosenthal, T. B., *J. Gerontol.*, 1950, v5, 112.

3. Lansing, A. I., Rosenthal, T. B., and Alex, M., *J. Gerontol.*, 1950, v5, 211.

4. Roberts, E., Ramasarma, G. B., and Lewis, H. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 237.

that all of the calcium was present in the form of $Ca_3(PO_4)_2$.

Preparation of heavy and light fractions. It was found that a separation of fractions having differing specific gravities could be achieved by differential centrifugation of finely ground elastin (Wiley mill) in sucrose solution (sp. gr. 1.30). Most of the material in samples of aortic elastin from very young individuals floated on the solution after centrifugation, while most of that from old individuals settled to the bottom. A sample of the suitably washed light fraction (young light) was prepared from several young aortas and some of the heavy material (old heavy) was prepared from old aortas. Both of these materials were analyzed for calcium and the contents of 18 amino acids.

Results. Calcium and nitrogen contents. The calcium and nitrogen contents of samples of elastin obtained from human autopsy specimens are given in Table I. The calcium content of the young aortic elastin was low, while that from the older specimens was markedly elevated. Calcium accumulation did not take place with age in the pulmonary elastin. The nitrogen contents of all of the samples were similar when corrections were made for the calcium content.

Paper chromatographic findings. Two dimensional chromatograms were made of acid hydrolysates of 15 samples of elastin from aortas of people of different ages. In all instances samples containing 77 μg of nitrogen were placed on paper for chromatography. The chief amino acids found in the samples were glycine, alanine, valine, the leucines, and proline. Also detected, but in much smaller concentrations, were tyrosine, serine, arginine, and glutamic and aspartic acids. The most striking visual difference between the chromatograms of aortic elastins from young and old specimens was the increased intensity of the spots corresponding to aspartic and glutamic acids in the latter. These findings made it seem advisable to perform quantitative determinations of the contents of some of the amino acids in the elastin preparations. As will appear in these quantitative analyses, other amino acids such as arginine, histidine, and threonine also increased with age.

TABLE I. Amino Acids, Calcium, and Nitrogen Contents of Elastin from Aortas and Pulmonary Arteries of Young and Old Individuals.

Sample	Age	Aspartic acid, g N/ 100 g N	Glutamic acid, g N/ 100 g N	Glycine, g N/ 100 g N	Proline, g N/ 100 g N	Leucine, g N/ 100 g N	Isoleucine, g N/ 100 g N	Valine, g N/ 100 g N	Amide NH_3 g N/ 100 g N	g Ca/ 100 g	g N/ 100 g† (corrected)
Young aortas	15	.24	1.32	26.6	11.4	4.71	2.22	13.6	1.43	.17	15.90
	17	.32	1.67	25.8	11.1	4.76	2.31	12.3	1.43	.16	15.75
	19	.31	1.43	27.0	10.8	4.94	2.27	13.2	1.43	.18	15.82
	20	.38	1.63	26.8	11.3	4.84	2.38	12.9		.88	15.35
	Mean	.31	1.51	26.6	11.2	4.81	2.30	13.2	1.43	.35	15.71
Old aortas	55	.65	2.13	25.6	10.4	4.92	2.46	11.4	1.83	4.58	16.22
	59	1.35	2.70	24.0	9.4	5.01	2.43	11.4	2.30	4.74	15.50
	65	.68	2.16	24.8	11.3	4.59	2.44	11.9	1.76	6.03	15.19
	65	1.70	3.06	22.6	9.4	4.94	2.60	10.7	2.40	6.61	15.37
	74	1.15	2.33	25.2	10.7	4.73	2.21	12.0	2.22	5.14	15.32
	75	.90	2.52	23.8	10.2	4.60	2.47	11.4	2.02	8.47	15.75
	Mean	1.07	2.48	24.3	10.2	4.80	2.43	11.5	2.09	5.93	15.56
Pulmonary arteries	15	.68	1.84	30.4	11.5	4.68	2.24	12.2	1.51*	.13	16.25
	26	.66	1.99	31.3	10.7	4.96	2.28	13.6	(1.38	.17	15.68
	77	.47	1.40	31.7	9.8	4.53	2.14	12.4	1.76)	.15	16.27
	92	.68	1.75	34.5	11.8	4.95	2.47	13.4		.64	15.78
	Mean	.62	1.75	32.0	11.0	4.78	2.28	12.9	1.51	.27	16.00

* These values were obtained from the analysis of 10 different samples of elastin prepared from pulmonary arteries ranging in age from 31 to 79 yr.

† Calculated on a Ca-free basis, assuming all of the Ca to be present as $Ca_3(PO_4)_2$.

Amino acid analyses. In Table I are shown the results of analyses for 7 amino acids. Highly significant increases in the contents of aspartic and glutamic acids were noted in the samples of old aortic elastin as compared to those of the young elastin, in confirmation of the chromatographic findings. There also were increases in the contents of amide nitrogen. However, there was a 4-fold increase in the excess of dicarboxylic acids over amide nitrogen, showing that the quantity of free carboxyl groups was increased significantly in the older specimens. The mean values for valine, proline, and glycine contents were somewhat lower in the older samples, while the leucine and isoleucine contents in the two groups were closely similar. In contrast to the findings for aortic elastin, the pulmonary elastin did not show increases in aspartic and glutamic acid contents with age. The other amino acids also did not change significantly. The contents of proline, leucine, isoleucine, and valine were virtually identical with those found in young aortic elastin. However, the contents of glycine and the dicarboxylic amino acids were higher in the pulmonary elastin. The quantity of free carboxyl groups was at least twice as great as that found in young aortic elastin, while the range showed some overlap with that of the old aortic elastin. In general, the values for the amino acids found in these human elastins were in the range of those previously reported for the aortic elastin of the pig, sheep and cattle(5) prepared by different methods.

TABLE II. Dicarboxylic Acid and Amide Contents of Elastin from Aortas and Pulmonary Arteries of Young and Old Individuals.

		Millimoles N/100 g total N		
		A	B	
Sample	Age	Aspartic + glutamic	amide	A-B
Young aortas	15	112	103	9
	17	142	103	39
	19	124	103	21
	Mean	126	103	23
Old aortas	55	199	130	69
	59	290	164	126
	65	203	126	77
	65	339	172	167
	74	248	158	90
	75	244	144	100
	Mean	254	149	105
Pulmonary arteries	15	180	108*	72
	26	189	108	81
	77	134	108	26
	92	174	108	66
	Mean	169		61

* See footnote * in Table I.

TABLE III. Amino Acid Composition of Young Light and Old Heavy Elastin.

Amino acid	Young "light"* g N per 100 g N	Old "heavy"† g N per 100 g N
Aspartic	0.38	1.11
Glutamic	1.83	3.01
Glycine	26.10	21.30
Proline	10.10	9.20
Leucine	4.52	4.76
Isoleucine	2.10	2.33
Valine	13.00	11.50
Alanine	23.18	21.58
Lysine	0.49	1.17
Arginine	1.78	4.35
Histidine	0.15	0.75
Cystine	0.06	0.10
Methionine	0.06	0.35
Phenylalanine	1.73	1.97
Tyrosine	1.45	1.76
Tryptophan	0.06	0.24
Serine	0.29	0.70
Threonine	0.65	1.13
Amide N	2.90	2.86
Total	90.83	90.17

* This preparation contained 1.14% calcium and 14.9% N.

† This preparation contained 6.39% Ca and 12.5% N.

Amino acids in young light and old heavy aortic elastins. In Table III are shown the contents of 18 amino acids in the light fraction of young aortic elastin and the heavy fraction of old aortic elastin prepared by suspension in sucrose in the manner described previously. In the case of the 7 amino acids which were determined on whole elastin (Table I), the differences observed between the young light and old heavy fractions were all similar to those found for the young and old whole elastin. The old heavy elastin showed increases in aspartic and glutamic acids and decreases in the contents of glycine, proline, and valine. There was also an increase in the number of free carboxyl groups in the old heavy sample. In addition, there was a decrease in the content of alanine. All of the other amino acids showed increases of varying degree in the old heavy elastin. Ap-

---

5. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v186, 549.

proximately 90% of the nitrogen of both samples was accounted for by the analyses. The light fraction contained 1.14% calcium while the heavy fraction contained 6.39%.

Discussion. The data in this paper show that the calcific changes previously observed to occur with age in the medial elastic tissue of the human aorta(2) are accompanied by changes in the amino acid composition of the elastin. It is doubtful that the shift in amino acid distribution represents a change with time in the amino acid composition of a pure protein. The elastin studied may be a mixture of two or more proteins, and the changes observed may be related to differences in the ratios of the separate constituents How such a change can come about is not yet known. It is possible that the proteins laid down later in life may be different from those formed earlier because they are formed under the influence of fibroblasts which themselves have undergone age changes. Previous reports(3) from this laboratory have emphasized the fact that the pulmonary artery is characterized by a low rate of calcification of the medial elastic elements. This investigation brings forth evidence that no significant amino acid changes occur with age in pulmonary elastin. On the other hand, the high rate of the elasto-calcinosis of the aorta can be correlated with a significant increase in dicarboxylic amino-acids.

It is tempting to speculate that the increased calcium binding capacity of the elastin from the older specimens is somehow related to the increased content of free carboxyl groups. Two mechanisms by which these changes could be related suggest themselves. It is possible that the calcium ion is bound by the free carboxyl groups of aspartic and glutamic acids and is then precipitated as calcium phosphate or some other phosphate-containing calcium complex because of the smaller dissociation constants of the inorganic complexes. The carboxyl group could then again become available for the binding of more calcium. Another possibility is that the calcium is bound by the free carboxyl groups, leaving one valence available for combining with phosphate, which could then bind more calcium, etc. No direct stoichiometric relationship exists between the free carboxyl groups and the calcium, the latter being found in great molar excess per unit weight of the old aortic elastin.

Summary. Comparative analyses of elastin from young and old aortas reveal a pattern of characteristic age changes consisting of increase in specific gravity, calcium, and free carboxyl groups. There is an underlying shift in relative amino acid composition. In contrast to the aorta, the pulmonary artery shows minimal age changes.

Received February 28, 1951. P.S.E.B.M., 1951, v76.

Fixed Rabies Virus in Blood Following Intracerebral Inoculation in Mice and Rabbits. (18605)

DOROTHY H. WONG AND JULES FREUND.

From the Division of Applied Immunology, The Public Health Research Institute of the City of New York.

There is little information on the distribution of viruses injected into the brain of animals. Schaeffer and Muckenfuss(1) studied the distribution of particulate matter introduced in the brain of monkeys by injecting India ink. They found only a very small part of the injected material at the site of its deposition. Schlesinger(2) observed that only 3 to 10% of the estimated amount of virus was recoverable from the infected brains of mice one hour after intracerebral inoculation of the Western equine encephalitis virus or

---

1. Schaeffer, M. and Muckenfuss, R. S., *Experimental Poliomyelitis,* 1940, p. 119, Sci. Press Printing Co., Pa.

2. Schlesinger, R. W., *J. Exp. Med.,* 1949, v89, 491.

the *E. coli* phage T1 or T7. He made no attempt to demonstrate the virus elsewhere. It was recently reported by Cairns(3) (after the completion of our observation) that C16 bacteriophage active against *Sh. flexner* and the W.S. strain of influenza virus can be demonstrated in the blood of mice within one minute after intracerebral inoculation. This is in harmony with the finding of M. Smith (4) who has shown that virus enters the blood stream within the first 2 hours after intracerebral inoculation of the poliomyelitis virus in mice.

From the point of view of the pathogenesis of rabies infection and the understanding of the test for immunity by intracerebral challenge, it is desirable to know whether the rabies virus behaves like those studied by Cairns and Smith. Since the rabies virus is larger than the viruses found to enter the blood stream following intracerebral inoculation, it seemed desirable to carry out similar experiments with rabies virus.

Materials and methods. Swiss white mice weighing from 8 to 10 g or rabbits weighing about 1500 g were used. Two strains of fixed rabies virus were employed, one received from the National Institute of Health, Bethesda, Md., and the other from the Illinois Department of Health. The LD_{50} of both strains was approximately 10^{-6}. One gram of mouse brain containing fixed rabies virus was suspended in 9 ml of distilled water containing 2% horse serum and 20 units of penicillin per 0.03 ml. The suspension was centrifuged at 1000 RPM for 10 minutes and the supernate used for injection in 3 groups of mice. Two other groups were injected with a 1:1000 dilution of the brain suspension. Mice were injected in one hemisphere of the brain receiving 0.03 ml of the virus suspension. Rabbits were similarly injected with 0.1 ml of the 1:10 brain suspension. To facilitate the collection of blood from mice heparin was injected subcutaneously in 4 groups of mice as indicated in Table I. At various intervals of time following the intracerebral inoculation, blood was collected aseptically either directly from the heart or by clipping the heart of the mouse. From the rabbits the blood was obtained either from the ear vein, the external or internal jugular vein, or the heart. The blood was centrifuged at 2000 RPM for 10 minutes. 0.03 ml of serum or plasma was injected intracerebrally into recipient mice. Suspensions of brains of recipient mice which developed symptoms of rabies were passed into new mice. Only when the latter mice died of rabies was a blood specimen considered positive. All mice were observed for a period of 3 weeks.

A total of 22 specimens of blood from 5 rabbits, taken during the first 3 hours following intracerebral inoculation, was tested; 12 from the ear vein, 4 from the external jugular vein, 4 from the internal jugular vein and 2 from the heart. One specimen of spinal fluid obtained 2 hours and 40 minutes after injection was tested.

Results. Table I gives the results of the 5 experiments in which the mouse was used. In all 5 experiments using mice, virus was demonstrated in the blood plasma or serum at some time during the first 2½ hours following intracerebral injection of the fixed rabies virus. There was no specific period of time during the 2½ hours in which every blood specimen tested for that period gave positive results. The blood serum or plasma of 15 host mice transmitted rabies to recipient mice; that of 24 mice failed to produce rabies. Only 2 blood specimens caused rabies in all recipient mice injected with an aliquot of that specimen; 13 transmitted the disease to one or more but not all of the recipient mice.

When rabbits were used as the host animal rabies virus was demonstrated in one of 5 rabbits. This was the specimen taken from the ear vein 3 hours after the intracerebral injection of the virus and produced rabies in one of 4 recipient mice receiving 0.03 ml of plasma. The one specimen of spinal fluid did not produce rabies in the recipient mice.

Discussion. The mechanism by which the virus reaches the blood stream is not known. It is possible that it enters the blood stream through blood vessels damaged by the injec-

---

3. Cairns, H. F., *Nature*, 1950, v166, 910.

4. Smith, M., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 88.

TABLE I. Fixed Rabies Virus in the Blood of Mice Following Intracerebral Injection.

	Host mouse				Blood recipient mouse	
	Rabies virus					
Exp.	Strain	Dilution	Heparin subcut., ml	Bleeding time interval after intracer. inoc.	No. inj.	No. rabies positive
1	I.D.P.H.	10^{-1}	0.1	2 min.	5	0
				15 "	5	0
				30 "	3	0
				40 "	5	1
				1 hr	4	0
				1 ", 15 "	5	0
				1 ", 20 "	5	0
				2 ",	3	1
				2 ", 5 "	2	0
				3 "	4	0
				3 ", 5 "	6	0
2	N.I.H.	10^{-1}	0.1	15 "	3	0
				30 "	3	3
				45 "	2	1
				1 hr	3	1
				1 ", 30 "*	7	0
				2 "	4	2
				2 ", 30 "	4	2
3	N.I.H.	10^{-3}	0.1	30 "	3	0
				45 "	4	0
				1 hr	2	0
				1 ", 30 min.	3	0
				2 "	5	0
				2 ", 30 "	2	1
4	N.I.H.	10^{-3}	0.1	15 "	5	0
				30 "	5	1
				45 "	4	1
				1 hr	2	0
				1 ", 30 min.	5	0
				2 "	1	1
				2 ", 30 "	7	1
5	N.I.H.	10^{-1}	—	15 "	5	1
				30 "	5	2
				45 "	4	0
				1 hr	4	1
				1 ", 30 "	4	0
				2 "	5	0
				2 ", 30 "	6	0

* 2 mice were used.

tion, but it seems more probable that it reaches the blood stream via the cerebrospinal fluid. The latter possibility is supported by the findings of Schaeffer and Muckenfuss(1) on the distribution of India ink following intracerebral injection in monkeys. They found "the distribution of the material following intracerebral deposition varied to some extent. In most cases, however, little of the material remained at the site of inoculation, but rapidly entered the cerebrospinal fluid either by seeping backward through the path of inoculation into the subarachnoid space or via the ventricles into the spinal canal." Insofar as the final deposition of the inoculum is concerned, they could draw no definite conclusion(5). It may be assumed that the rabies virus having reached the cerebrospinal fluid passes through the arachnoid villi into the blood stream. It is stated by Best and Taylor(6), however, that true solutions and colloids pass through the arachnoid villi but particulate matter does not. If we are correct in our assumption, it is of interest that a virus as large as the fixed rabies virus, which

---

5. Schaeffer, M. and Muckenfuss, R. S., *Am. J. Path.*, 1938, v14, 227.

6. Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, 1950, p. 1069, Williams and Wilkins Co., Baltimore.

has been estimated to be 0.1 to 0.21 micron (7-9) in size, can pass through the arachnoid villi.

At the outset of the experiments we had expected that after intracerebral injection the virus would be readily demonstrable in the blood of the rabbit, in blood samples taken from the internal jugular vein. This assumption was not confirmed.

Summary. Fixed rabies virus was recovered from the blood stream of the mouse and rabbit during the first 3 hours following intracerebral inoculation.

Received March 1, 1951. P.S.E.B.M., 1951, v76.

---

7. Levaditi, C., Paic, M. and Krassnoff, D., *C. R. Soc. Biol.*, 1936, v123, 866.

8. Yaoi, H., Kanazawa, K., and Sato, K., *Jap. J. Exp. Med.*, 1936, v14, 73.

9. Galloway, I. A., and Elford, W. J., *J. Hyg.*, 1936, v36, 533.

Metabolism of Radioactive Vitamin B_{12} by the Rat.* (18606)

KENDALL W. BARBEE AND B. CONNOR JOHNSON.

From the Division of Animal Nutrition, University of Illinois, Urbana.

Radioactive vit. B_{12} labeled with Co^{60} has been prepared by Rosenblum *et al.*(1) by fermentation. We have used this compound[†] to study the metabolism of vit. B_{12} in the rat. Chow *et al.*(2) have reported that approximately 80% of the vitamin is excreted in the urine within 24 hours when injected subcutaneously, while at least 270 γ/300 g rat is needed to cause the excretion of the vitamin in the urine when administered orally.

In the present experiment we have studied the retention and excretion of radioactive vit. B_{12} when given orally and when given by injection and also the form of the Co (whether as vit. B_{12} or other) stored and excreted.

Experimental. Four adult white rats were used for this experiment. Rats A and B were used for qualitative work, and rats C and D for quantitative work. One ml of Co^{60} labeled vit. B_{12} solution containing approximately 20 γ of vit. B_{12}, approximately 1.3 microcuries of radioactivity, was administered to each rat. The vit. B_{12} was injected subcutaneously into rats A and C and was given orally with the dry ration of ground checkers fed to rats B and D. The excreta were collected at 24-hour intervals for 3 days, at which time the animals were sacrificed. Paper strip chromatograms were run on the urine, feces, and extracts of the kidneys, liver and spleen of rats A and B. The solvent system was that used by Winsten and Eigen(3). Radioautographs were made of these chromatograms and of a pure radio-vit. B_{12} chromatogram. A paper strip chromatogram was also

TABLE I. Radioautographs of Paper Strips.

	R_f (of spot)
Cobalt chloride	.0
Vit. B_{12}	.05
Vit. B_{12b}	.08 to 0.13
Rat A (given B_{12} by inj.)	
24-hr urine	.13 (B_{12b})
Kidney	.095 (B_{12b})
24-hr feces	.0 (Co^{60++})
Liver	No spot
Rat B (given B_{12} orally)	
24-hr urine	.07 to 0.10 (B_{12b})
24-hr feces	0.0; .074 (Co^{60++} and B_{12})
Kidneys	No spots
Liver	
Spleen	

---

* Supported in part by a grant-in-aid from Merck and Co., Inc., Rahway, N. J., and in part by a grant from the U. S. Atomic Energy Commission under Contract No. AT (11-1)-67, Animal Science Project 2.

1. Charet, L., Rosenblum, C., and Woodbury, D. T., *Science,* 1950, v111, 601.

[†] We wish to thank Dr. Charles Rosenblum of Merck and Co., Inc., Rahway, N. J., for the generous gift of 20 μc of radioactive vitamin B_{12}.

2. Chow, B. F., Barrows, L., and Lang, C., *J. Nutrition,* 1950, v42, 405.

3. Winsten, W. A., and Eigen, E., *J. Biol. Chem.,* 1949, v181, 109.

TABLE II. Distribution of the Administered Radio-Vit. B_{12}. 1 ml of vit. B_{12} solution gave 285000 ± 2850 counts/min.

Rat	Count/min.		% of total given	
	C (inj.)	D (orally)	C (inj.)	D (orally)
24-hr urine	265650 ± 2657	1500 ± 30	93.21	.53
48-hr ”	2375 ± 48	300 ± 24	.83	.11
72-hr ”	1500 ± 30	300 ± 24	.53	.11
24-hr feces	2200 ± 44	253825 ± 2538	.77	89.06
48-hr ”	1950 ± 39	6325 ± 64	.68	2.22
72-hr ”	1185 ± 24	1875 ± 38	.42	.66
Feed dish	—	450 ± 36	—	.16
Intest. cont.	1365 ± 27	1625 ± 33	.48	.57
Liver	1300 ± 26	1250 ± 25	.46	.44
Kidneys	1475 ± 30	1775 ± 36	.52	.62
Spleen	925 ± 19	800 ± 32	.32	.28
Blood	802 ± 16	625 ± 50	.28	.22
Body	—	5400 ± 216	—	1.89
Total	280727 ± 2960	276050 ± 3146	98.5 ± 1.04	96.86 ± 1.1

run on a non-radioactive $CoCl_2$ solution, the Co being detected by color. The feces, intestinal contents, blood, spleen, liver, and kidneys of rats C and D were ground and digested for 3 days at 37° with trypsin and pancreatin at pH 8.0. The urine samples and extracts of feces and tissues were concentrated to 25 ml. One ml of each sample and one ml of the vit. B_{12} solution (diluted 1:1000) were taken for counting. The samples were dried in aluminum counting pans under infrared light and counted in a "Q-gas" counter.‡ The error due to self-absorption was overcome by using an aluminum shield to absorb the β-rays, the γ-rays only being counted.

Results. The Rf values of the spots observed on the radioautographs of the paper strip chromatograms are given in Table I. The distribution of the radioactive cobalt between urine, feces and various tissues of the rat following oral or intramuscular administration of radiovit. B_{12} is given in Table II.

Discussion. From Table II it is observed that when the radiovitamin B_{12} is given orally, approximately 92% of the radioactivity appears in the feces within 72 hours, while when given by injection approximately 95% was excreted in the urine in the same period of time. When injected there is a small excretion of Co^{60++} in the feces, and when given orally there is a small excretion of vit. B_{12} in the urine. On the basis of the small amount of Co^{60} excreted into the feces following injection of radiovit. B_{12} it is apparent that the radioactivity of the feces following oral ingestion is due primarily to unabsorbed vit. B_{12}. However, on the basis of the chromatogram it appeared that about 60% of this unabsorbed vit. B_{12} had been decomposed by bacterial action to yield radiocobalt.

Apparently the Co^{60++} excreted in the feces following injection is an end product of vit. B_{12} metabolism in the body. The results indicate that some vit. B_{12} is excreted in the urine even when a small dose is given orally but that most of it is unabsorbed. On the basis of the rat kidney chromatogram (Fig. 1) it appears that the Co^{60} is stored in the tissues as vit. B_{12}, the small amount of ionic cobalt formed by metabolism being excreted via the intestine.

Summary and conclusions. Radioactive vit. B_{12} has been given orally or subcutaneously to 4 adult white rats. The radioactivity (entirely B_{12}) was excreted primarily in the urine following injection, while the radioactivity (B_{12} + inorganic cobalt) was excreted primarily in the feces following oral administration.

Received March 5, 1951. P.S.E.B.M., 1951, v76.

---

‡ Nuclear Instrument and Chemical Corporation, Chicago, Ill.

Effect of Cortisone on Growing Bones of the Rat. (18607)

RICHARD H. FOLLIS, JR.
From the Department of Pathology, Johns Hopkins University School of Medicine, Baltimore.

Because of the profound changes which may be encountered in the skeleton in cases of Cushing's syndrome, particularly in the infant(1), we have felt it was desirable to determine whether such lesions could be reproduced experimentally with adrenal cortical hormone. Wells and Kendall(2) have reported a retardation of growth in rats treated with Compound E. Winter *et al.*(3) have administered 3 mg cortisone daily to rats weighing 200 g. Sections of the ribs and tibiae of such animals revealed a reduction in the width of the epiphyseal cartilage but no rarefaction of cortical or cancellous bone.

Experimental. Cortisone acetate* was administered subcutaneously each day to male rats (McCollum strain) weighing 40-50 g at the beginning of the experiment. The following amounts of cortisone in mg per kilo body weight per day were employed: 0.25, 2.5, 10.0, 20.0, 40.0, 50.0, 75.0, and 100.0. There were 6 to 8 rats in each group. To control those groups which failed to gain the food intake of normal rats was restricted so as to give comparable growth curves. At varying intervals animals were sacrificed; the ribs and long bones were fixed in 80% alcohol or neutral 4% formalin, dehydrated, embedded in paraffin or nitrocellulose and stained with hematoxylin-eosin or eosin-toluidine blue.

Results. When daily doses of cortisone amounting to 0.25, 2.5, 10.0 and 20.0 mg per kilo were administered to young rats there was no appreciable effect on their growth over a period of 3 weeks as compared with control animals of the same weight. However, when 40 or 50 mg per kilo were administered, after an initial gain for 1 or 2 days the growth curves flattened out and remained so during a 3 to 4 week interval. Amounts of cortisone greater than this led to a prompt plateauing of the growth curve followed by a gradual loss of weight.

Examination of the skeletal tissues revealed no change in the groups receiving .25 to 20 mg per kilo, *i.e.* those whose growth was unaffected. In the bones of the rats receiving 40 or 50 mg per kilo profound alterations were found. After being treated for 3 days there was a definite narrowing of the epiphyseal cartilage due in large part to a reduction in the number of hypertrophic cells, but also as a result of retardation in the proliferation of undifferentiated cells. More prominent was the presence of an increased amount of calcified cartilagenous matrix encased in bone beneath the epiphyseal cartilage. As the duration of treatment continued this zone increased in width so that after 3 weeks there was a broad zone of dense interlacing trabeculae each with a central core of cartilage matrix encased in bone. There did not appear to be any excess number of osteoblasts nor were osteoclasts present in any conspicuous form. There was no increased activity of the periosteal cells nor was there any evidence of increased or decreased endosteal bone formation in the cortex of the shaft. In comparison with the changes noted above at the cartilage-shaft junction in rats whose growth was inhibited by caloric restriction, although the width of the epiphyseal cartilage plate was reduced, there was rarefaction of the underlying spongiosa. When rats to which cortisone had been administered for 2 weeks were then allowed to go untreated there was a resumption of normal growth activity of the cartilage. The mass of cartilagenous matrix encased in bone moved away from the cartilage as the latter proliferated and was then slowly destroyed. Animals receiving 75 or

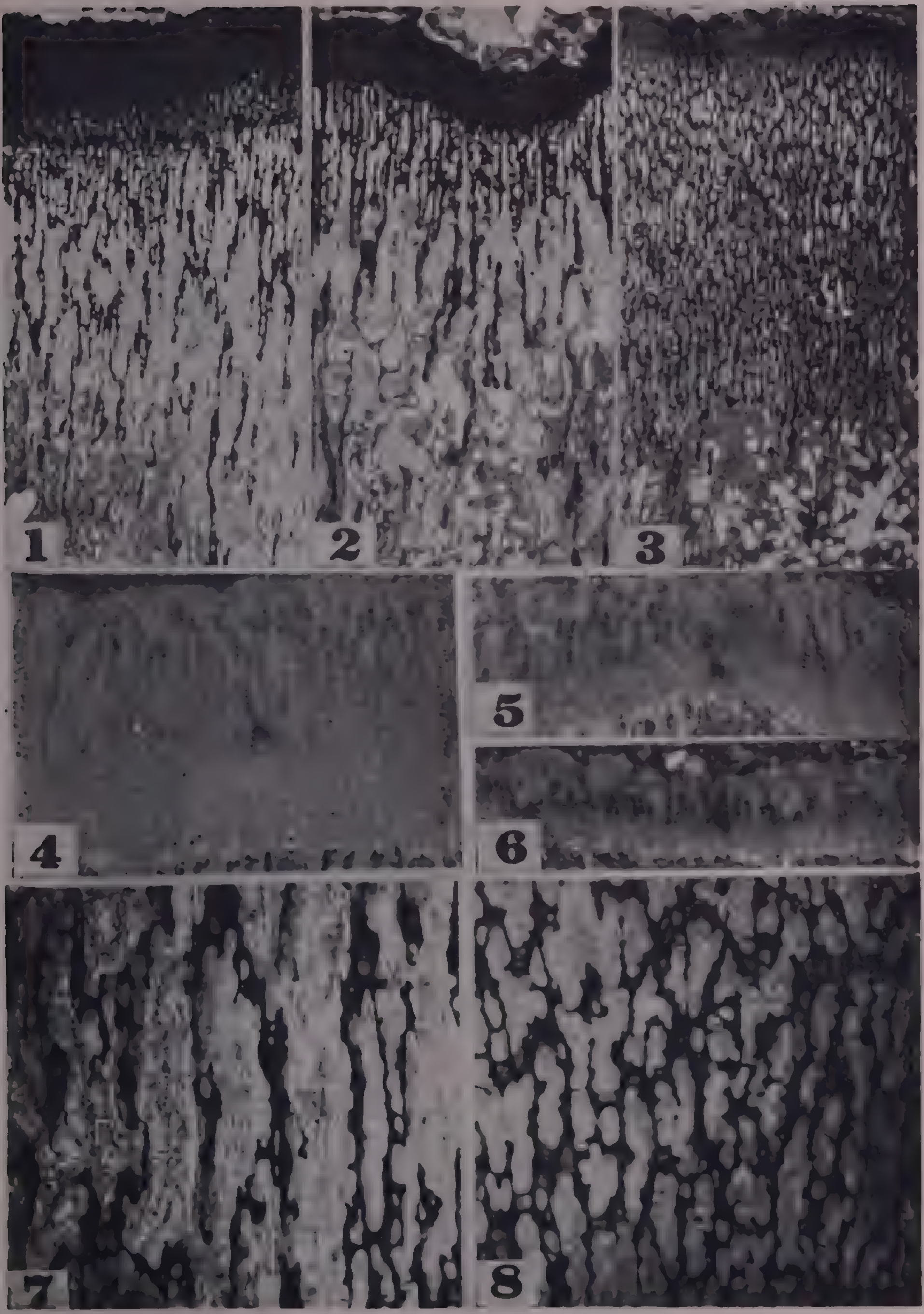
---

1. Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.*, 1951, v88, 440.

2. Wells, B. B., and Kendall, E., *Proc. Staff Meetings Mayo Clin.*, 1940, v15, 324.

3. Winter, C. A., Silber, R. H., and Stoerk, H. C., *Endocrinol.*, 1950, v47, 60.

* The cortisone acetate (Cortone) was furnished through the kindness of Merck and Co., Rahway, N. J.



All sections stained with eosin-toluidine blue.

FIG. 1. Normal tibia of young rat (×32).

FIG. 2. Tibia of rat whose diet had been restricted calorically so weight gain was similar

to rat shown in Fig. 3. Note great reduction in width of epiphyseal cartilage and fewer trabeculae in shaft (×32).

FIG. 3. Rat treated with 50 mg cortisone for 3 weeks. Note great narrowing of cartilage and increased density of zone made up of cores of calcified matrix encased in bone. Fig. 1, 2, and 3 are all of same magnification (×32).

FIG. 4. Higher power of epiphyseal cartilage of normal rat (×64).

FIG. 5. Higher power (same magnification as Fig. 4) of epiphyseal cartilage of rat shown in Fig. 2 (×64).

FIG. 6. Higher power (same magnification as Fig. 4 and 5) of same cortisone treated animal shows in Fig. 3 (×64).

FIG. 7. High power view of spongiosa of normal rat (×64).

FIG. 8. Higher power view (same magnification of rat shown in Fig. 7) to show increased amount of persisting calcified matrix encased in bone (×64).

100 mg cortisone daily per kilo whose growth curves showed a drop did not exhibit the above change, due apparently to complete cessation of growth activity.

Discussion. There would appear to be two possible ways to explain the pathogenesis of the changes noted above: increased osteoblastic activity or decreased osteolytic activity or a combination of both. As far as the former is concerned there does not appear to be an increased number of osteoblasts about the trabeculae of the spongiosa beneath the atrophic epiphyseal cartilage. There is no excessive formation of cortical bone, either periosteal or endosteal. The width of the zone of increased density corresponds with the interval in which the animal was treated with cortisone so that one must assume that normal osteoblastic growth sequences have taken place. On the other hand the persistence of large amounts of calcified cartilagenous matrix encased in bone is certainly most abnormal so that the conclusion seems unescapable that there is an inhibition or retardation in normal osteolytic sequences. Such changes are, of course, not those which may be encountered as a result of caloric restriction or deficiency of one or more of the forty-odd essential nutrients. Under such circumstances there is narrowing of the epiphyseal cartilage together with rarefaction of the spongiosa beneath(4).

The change produced in these bones by cortisone is most reminiscent of alterations which may be encountered in the growing rat when large amounts of estrogen are administered. Some years ago the conclusion was reached(5) that the increased density at the growing ends of the bones of estrogen treated rats was due to some derangement in destructive activity. This interpretation has been upheld by others(6). The mechanism is, of course, unknown. In view of the peculiar effect of estrogen on the overproduction of endosteal bone in the mouse it will be most interesting to study the effects of cortisone in this species.

Summary. When cortisone acetate in appropriate doses is administered to growing rats, a dense zone composed of spicules of calcified cartilagenous matrix encased in bone appears at the growing ends of the bone. The width of this zone may be correlated with the duration of administration of the hormone. It is concluded that this area of increased density results from a disturbance in normal osteolytic activity.

4. Follis, R. H., Jr., *J. Mount Sinai Hosp.*, 1949, v16, 1.

5. Day, H. G., and Follis, R. H., Jr., *Endocrinol.*, 1941, v28, 83.

6. Urist, M. R., Budy, A. M., and McLean, F. C., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 324.

Received March 5, 1951. P.S.E.B.M., 1951, v76.

Blocking Action of Tetraethylammonium on Lobelin-Induced Thoracic Pain. (18608)

JAMES E. ECKENHOFF AND J. H. COMROE, JR. (Introduced by D. L. Drabkin)

From the Departments of Anesthesiology, Physiology and Pharmacology, Graduate School of Medicine, Harrison Department of Surgical Research, University of Pennsylvania.

The actions of tetraethylammonium (TEA) are complex. In general it blocks all the stimulatory nicotinic actions of acetylcholine (ACh) without interfering with the muscarinic effects of the drug. Thus it blocks the stimulatory action of ACh upon sympathetic and parasympathetic ganglion cells and upon motor end plates in skeletal muscles; presumably on this account it produces "ganglionic blockade" of motor autonomic fibers and depression at the neuromuscular junction. The effects of TEA upon the sensory system have been reviewed by Moe and Freyburger(1). Here again, TEA seems to act specifically upon sensory receptors stimulated by ACh (nicotinic action), nicotine or its congener, lobeline. Receptors so affected include some carotid and aortic chemoreceptors(2), those which initiate pilomotor and sudomotor cutaneous axone reflexes(3,4), and certain pain and position receptors(5).

We have observed another instance in which TEA blocks sensation. We have noted that lobeline, injected rapidly intravenously, for the purpose of measuring arm to aorta or carotid circulation time, usually produced substernal burning and distress associated with explosive cough within 8 seconds after injection. Since these sensations precede aortic or carotid chemoreceptor-induced hyperpnea by five to ten seconds, we believe that they are due to stimulation of sensory receptors supplied by the pulmonary circulation, probably in the pleura or respiratory tract. In the present study we have found that this type of chemically induced pain is susceptible to the blocking action of TEA.

We injected 5.0-7.5 mg of lobeline (free from preservative) into 7 patients who were to receive TEA for the treatment of peripheral vascular disease. In all except one, the dose was insufficient to cause hyperpnea. Six of these, however, experienced substernal burning discomfort, which disappeared in less than 2 minutes, and a paroxysm of coughing of about 30 seconds duration (Table I). After 10 to 15 minutes, each of these 6 was given 125-200 mg of TEA by slow intravenous injection. Ten to 15 minutes later, lobeline was repeated in dosage similar to or larger than that previously injected. This second injection produced either no reaction or a markedly diminished one. Since repeated injections of lobeline may result in paralysis of structures stimulated by earlier doses, we repeated the injection of lobeline in patient F. S. (Table I) without the intervening injection of TEA; there was no diminution in the burning sensation and cough.

Discussion. TEA does not seem to be an analgesic or narcotic in the usual sense of the word, since it does not relieve all types of pain, does not alter the threshold for burning pain, and does not alter reaction to pain or affect pain centers(5). When pain is due to increased activity of smooth muscle (in blood vessels or in the gastrointestinal tract) or of glands (gastric acid secreting glands), TEA produces relief by effecting autonomic blockade. However, some sensory receptors appear to be blocked directly and selectively by TEA. In all instances described to date (aortic and carotid chemoreceptors, cutaneous and thoracic receptors), these appear to be chemical receptors, all of which have the property of being stimulated by nicotine, lobeline or ACh.

We have been unable to localize these thor-

---

1. Moe, G. K. and Freyburger, W. A., *Pharmacol. Rev.*, 1950, v2, 61. (*J. Pharmacol.*, April, 1950).

2. Moe, G. K., Capo, L. R., and Peralta, R. B., *Am. J. Physiol.*, 1948, v153, 601.

3. Coon, J. M., and Rothman, S., *J. Pharm. Exp. and Therap.*, 1941, v73, 1.

4. Janowitz, H. and Grossman, M. I., *Science*, 1949, v109, 16.

5. Sonnenschein, R. R., Jenney, E. R., and Pfeiffer, C. C., *J. Applied Physiol.*, 1949, v2, 141.

TABLE I.

Patient	1st inj. lobeline, mg	Response	TEA, mg	2nd inj. lobeline, mg	Response
E.M.	5	Coughing 2×. Substernal burning beginning in 11 sec.	200	5	Very slight burning in 50 sec.
A.	7	"Dry sensation of throat." Cough	175	7	None
M.I.	6	Pronounced cough. Substernal burning	150	6	No cough. Slight burning
A.D.	6	Substernal burning. Repeated coughing. Tachypnea	125	7	Cough one time 12 sec. None other
L.M.	7.5	Substernal oppression. Repeated cough	200	8	None
F.S.	6	Substernal burning. Paroxysmal coughing. Sulfur taste	125	7	Cough one time. Sulfur taste
F.S.	6	Substernal burning. Paroxysmal coughing	—	6	Substernal burning. Paroxysmal coughing

acic receptors. Experiments performed upon lightly narcotized dogs, in which lobeline was injected into the pleural cavity, the peritoneum or pulmonary artery or sprayed into the trachea, have not been rewarding because of the lack of objective indications of the experience of pain. Analogous studies have not been attempted in man.

Nevertheless, we suggest tentatively that the receptors may be in the visceral pleura because of two previous observations: (a) McLaughlin(6) has reported the presence in the visceral pleura of the cat of specialized cells which bear a strong resemblance to Pacinian corpuscles in the mesentery; it is known that the latter are stimulated by nicotine(7) and therefore presumably by its congener, lobeline. (b) Israel and associates(8) have observed that some types of pleural pain are relieved promptly by the intravenous injection of TEA.

We do not know what might constitute the natural stimulus to these receptors though we suspect that it is chemical (local chemical products of inflammation or of metabolism) rather than mechanical. However, TEA seems to have the property of blocking these receptors selectively without interfering with adjacent sensory receptors in the chest wall.

Direct proof of the hypothesis that these are pleural receptors would require that characteristic pleural pain be reproduced by the injection of a chemical excitant into the pleural cavity and be blocked by TEA administered by the same route. This we have not attempted in man.

Summary. The hypothesis is offered that specialized pain receptors exist in the visceral or parietal pleura and that TEA relieves certain types of thoracic pain by blocking these receptors. Attempts to demonstrate these receptors in lightly narcotized animals have been unsuccessful. However, it has been demonstrated that lobeline injected intravenously into man will produce substernal burning and coughing, which can be blocked by the prior injection of TEA. The significance of these observations is discussed.

---

6. McLaughlin, A. I. G., *J. Physiol.*, 1933, v80, 101.

7. Brown, G. L. and Gray, J. A. B., *J. Physiol.*, 1948, v107, 306.

8. Israel, H. L., Keeter, E. L., Urbach, F. E., and Willis, W. D., *N. Eng. J. Med.*, 1949, v241, 738.

Received March 5, 1951. P.S.E.B.M., 1951, v76.

Cell Volume Determinations with Evans Blue (T1824). (18609)

W. M. McCord and Humphrey K. Ezell.

From the Department of Chemistry, Medical College, State of South Carolina, Charleston, S. C.

The determination of blood volume with Evans Blue(1) involves the determination of red cell volume. The value for the red cell fraction usually is found by use of the hematocrit, which may present certain disadvantages. Colorimetric methods have been published, and are in use, in which known amounts of Evans Blue, often as a dry residue in a tube, are added to known amounts of blood. After dispersion, and solution of the Evans Blue in the blood, the red cells are spun down, the absolute dye concentration in the plasma determined colorimetrically, and the relative volumes calculated. By this method, the hematocrit, with its various inaccuracies, is not used; but accurate, and often laborious, measurements of the Evans Blue to be added, must be made. Observation of spectral transmittance curves of hemoglobin and of Evans Blue solutions indicates that a large difference occurs in optical density of solutions of hemoglobin and of Evans Blue in the spectral region of 600-640 mμ (Fig. 1). It is on this basis that previous methods have been developed, in that small amounts of hemolyzed blood would have but little effect upon optical density measurements of Evans Blue. On the same basis it should be practicable to determine Evans Blue concentration in the presence of large amounts of hemolyzed blood. By making measurements of relative dye concentration upon a single sample of blood, that is, upon the plasma and upon the hemolyzed whole blood, one should be able to calculate relative volumes of plasma and whole blood from relative dye concentrations. The use of relative dye concentrations would make it unnecessary to know (to weigh or to determine) absolute amounts of dye in calculating cell volume. This method would then make it possible to determine cell volumes upon samples of blood taken for routine blood volume determinations using Evans Blue. Also, by addition of approximate quantities of Evans Blue to blood *in vitro,* the cell volume could be accurately measured. Assuming the validity of Beer's Law, the following relationships will pertain when working with a sample of blood containing Evans Blue, and a blank sample of the same blood containing no dye.

Plasma dye conc. = K (optical density of dyed plasma—optical density of blank plasma).

Hemolyzed whole blood dye conc. = K (optical density of dyed blood—optical density of blank whole blood), and (conc. of dye in whole blood)/(conc. of dye in plasma) = (vol. plasma)/(vol. of whole blood) = plasma fraction and cell fraction = 1—plasma fraction.

Procedure. Blood samples, oxalated or heparinized, are obtained in the usual manner for determination of blood volume using Evans Blue, that is, an initial sample is procured, a known quantity of Evans Blue solution, usually 2 ml of 1%, is injected by vein, and, usually 10 or 20 minutes later, a sample or series of samples is obtained. Of the initial blood sample, containing no Evans Blue, 0.3 ml are added to 2.7 ml of distilled water. The remainder is centrifuged and 0.3 ml of plasma are added to 2.7 ml of distilled water. The sample containing Evans Blue is treated in a like manner. After standing for 10 minutes to allow complete hemolysis to occur, the hemolyzed samples are centrifuged to remove suspended cell stroma. With the diluted initial plasma sample as a blank the optical density of the other three solutions is determined at 625 mμ. (In this case with a Beckman D.U. spectrophotometer). In the event that optical densities are found to be too high for accurate reading, the solutions may be diluted further or the wave length at which the readings are made may be increased to 640 mμ without invalidating the method of calculation. Plasma fraction =

---

1. Shohl, Alfred T. and Thomas, H. Hunter, *J. Lab. Clin. Med.,* 1941, v26, 1829.

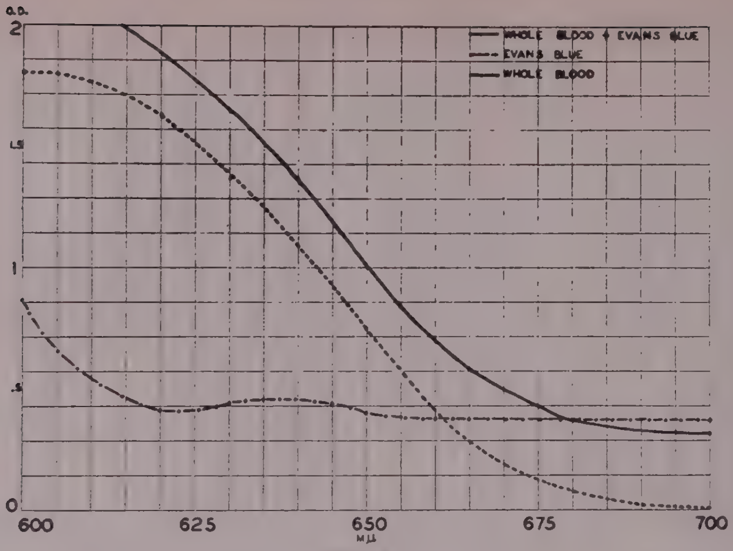


FIG. 1.

$(\text{O.D.}_1 - \text{O.D.}_2)/(\text{O.D.}_3)$ where O.D._1 = optical density of hemolyzed whole blood containing Evans Blue; O.D._2 = optical density of hemolyzed initial blood sample; O.D._3 = optical density of plasma containing Evans Blue (using dye free plasma as a blank).

Cell fraction = 1—plasma fraction. Routine blood samples, oxalated or heparinized, may be treated in the same way *in vitro*. The cell fraction is determined by dividing the blood sample into two parts and adding approximately 0.02 ml of 0.1% Evans Blue solution for each ml, to one part of the divided blood sample. A procedure, identical with that just described, is followed and cell fractions calculated.

TABLE I.

Cell vol. colorimetric method	Cell vol. corrected hematocrit	Difference
.375	.360	+.015
.392	.420	—.028
.421	.423	—.002
.288	.290	—.002
.415	.432	—.017
.395	.404	—.009
.412	.395	+.017
.427	.433	+.004
.334	.342	—.008
.338	.342	—.004
.427	.424	+.003

Results obtained by this colorimetric method were compared with those obtained by use of the hematocrit. The hematocrit was spun for 15 minutes at 3000 R.P.M. and a factor of .92 was used to correct for the volume of plasma trapped between the packed red cells. Experimental results are presented in Table I.

Summary. A colorimetric method is presented for the determination of cell fractions of blood. Accurate measurements of dye are unnecessary. The procedure may be applied *in vitro* or *in vivo*, in which case the routine samples drawn for determination of blood volume may also be used for the determination of the cell fraction.

Received March 5, 1951. P.S.E.B.M., 1951, v76.

Influence of Age, Body Weight, and Sex on Susceptibility of Mice to the Lethal Effects of X-radiation.* (18610)

HERBERT L. ABRAMS.† (Introduced by Henry S. Kaplan)

From the Department of Radiology, Stanford University School of Medicine, San Francisco.

The effects of age, body weight, and sex on susceptibility to radiation mortality have not been thoroughly explored. Furth(1) mentioned that, among a series of mice exposed to 300-400 r, mortality was greatest in young animals aged about 5 weeks at the time of irradiation. Quastler(2) drew a hypothetical curve of the age-survival time relationship based upon his experimental age-weight and weight-survival time relationships. This curve indicated that among mice 25 days or older, increasing age was accompanied by increased resistance to irradiation. He suggested that this was a reflection of the increasing body weight found in older age groups. His actual observations, however, were too few and too scattered to delineate a definitive age-survival time relationship. He also stated that "infant mice survive a given dose considerably longer than adult mice."

In the present experiments, the effects of age, weight and sex on mortality and survival time in irradiated mice were investigated.

Methods. Strain C57 black mice received a single exposure of 550 r measured in air.‡ They were divided into 6 groups of about 50 mice each, one group being irradiated at an average age of 1, 15, 30, 45, 60 and 90 days, respectively. Litters were distributed among all groups. The over-all sex distribution was approximately equal, although there were variations among the groups. All mice were weaned at the age of 30 days and subsequently maintained on Purina laboratory chow and water *ad libitum* under identical laboratory conditions. Body weight was determined on the day of irradiation.

Results. (1) *Age.* Susceptibility of mice to the lethal effects of irradiation is strikingly influenced by age at the time of exposure (Fig. 1, Table I). During a 30-day observation period, mortality was low in the 1- and 15-day-old groups, very high in the 30-day-old group, and decreased progressively with age thereafter. When the observation period was extended to 60 days, the mortality of the 1-day-old mice increased significantly (Fig. 1). In general, survival time was inversely related to mortality. Among the mice irradiated at the age of 1 and 15 days, a majority of deaths occurred within 2 weeks after weaning (Fig. 2). Surviving mice of the 1-day-old group exhibited marked stunt-

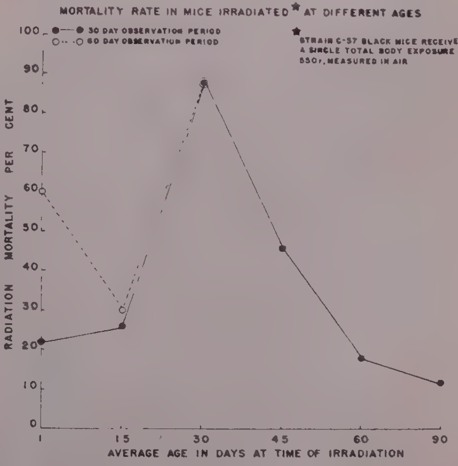


FIG. 1.

---

[*] This investigation was supported by a grant to Dr. Henry S. Kaplan from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[†] Trainee, National Cancer Institute.

1. Furth, J., and Furth, O. B., *Am. J. Cancer,* 1936, v28, 54.

2. Quastler, H., *Am. J. Roentgen. and Rad. Ther.,* 1945, v54, 457.

[‡] Physical factors: 120 KVP, 9 ma, 30 cm focus skin distance, 0.25 mm Cu and 1.0 mm Al added filtration. HVL 0.36 mm Cu, output 32.2r per minute. Groups of 8 mice were irradiated in a flat, round lucite container. In an effort to ascertain whether increased scatter might alter the results among heavier mice, readings were made with an ionization chamber under conditions duplicating those of the experiment. No significant difference in dose within the lucite container could be found among the various age groups.

TABLE I. Mortality and Average Survival Time in Mice Irradiated at Different Ages.

Avg age at time of irradiation, days	No. of mice irradiated	Deaths: 30-day observation period No.	%	Deaths: 60-day observation period No.	%	Avg survival time (days): 30-day observation period	Avg survival time (days): 60-day observation period
1	58	13	22.5	35	60	26.5	40
15	57	15	26.5	17	30	26.5	47
30	59	53	90	53	90	12	15
45	65	30	46	30	46	22	37.5
60	52	10	19	10	19	27	51
90	45	6	13.5	6	13.5	28	54

ing, physiological immaturity, and neuromuscular incoordination.

(2) *Weight.* Mortality is plotted against body weight in Fig. 3. A total of 316 mice were used, each point representing at least 5 mice. No correlation between mortality and body weight was found. Mortality tended to vary with age rather than with body weight. Striking evidence of the absence of a mortality-weight correlation is seen in the group of mice weighing 17 to 18 g. Among 30-day-old mice of this weight, mortality was 83%, while 45-day-old mice of the same weight had a mortality of only 22%. Analysis of unpublished data for a large single age group (255 mice aged 30 days at time of irradiation) likewise revealed an absence of relationship between weight and mortality.

(3) *Sex.* With the possible exception of 90-day-old mice, in which the mortality among females was suggestively higher than among males, sex did not influence susceptibility significantly.

Discussion. In general, young, rapidly growing tissues are thought to be most susceptible to the effects of irradiation. Yet many mice irradiated at 1 and 15 days of age were capable of surviving a 30-day post-irradiation period, while 30-day-old mice suffered a high mortality. This result is contrary to what might be expected on the basis of a simple relationship to general tissue maturity or to rate of body growth.

Some other explanation must therefore be sought for this age effect. That nutritional disturbances, physiological state, and other factors associated with weaning may be operative is suggested by the high incidence of deaths soon after weaning in the 1- and 15-day-old groups. In general, mice subjected to a single whole body exposure of 550 r develop anorexia and diarrhea and lose weight prior to death. Within 3 days after irradiation, many mice of the 30-day-old group were too weak to obtain food or water; when food was presented, they ate little. Attempts to substitute bread and milk for Purina laboratory chow met with the same response. In contrast, mice irradiated at the age of 1 day continued to suckle until the time of weaning. Adequate protein intake has been shown to result in diminished susceptibility to total body irradiation in rats(3). Thus, their relative nutritional state may explain in part the difference in response to irradiation of 1- and 30-day-old mice. Other hypothetical protective influences associated with suckling include: (a) preservation of fluid balance; (b) an anti-hemorrhagic factor in mouse milk to counteract increased plasma concentrations of anti-coagulant substances after irradiation (4); (c) a factor stimulating bone marrow recovery(5); (d) factors increasing resistance to infection, and (e) hormonal factors. Proximity of suckling mice to the mother affords additional warmth which may also be supportive(6). These possibilities will require further experimental investigation.

---

3. Jennings, F. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 487.

4. Allen, J. G., and Jacobson, L. O., *Science,* 1947, v105, 388.

5. Jacobson, L., Marks, E. K., Gaston, E. O., Robson, M., and Zirkle, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 740.

6. Smith, W. W., Highman, B. J., Mitchell, J. R., and Blount, H. C., Jr., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 498.

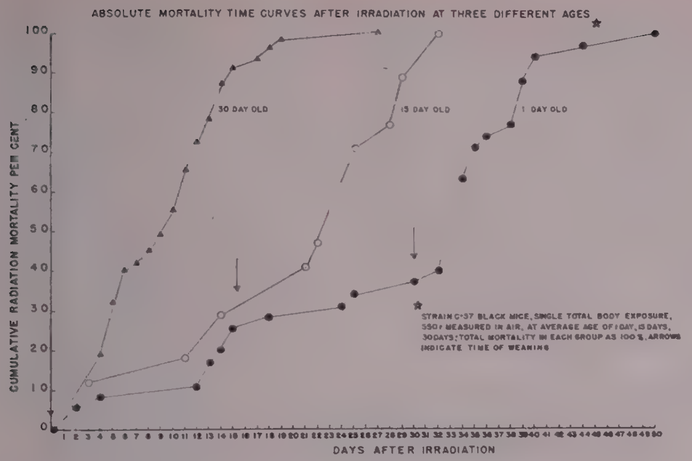


FIG. 2.

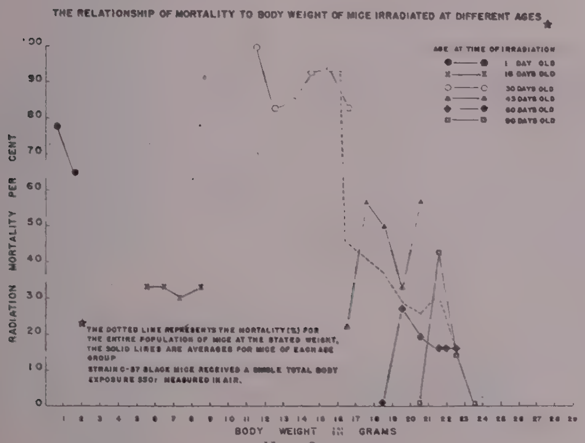


FIG. 3.

Conversely, emphasis may be placed upon the heightened susceptibility of the 30-45-day-old groups. It is noteworthy that this is the age of puberty in mice, and the endocrine upheaval which takes place during this period may well be an important factor. Support for this hypothesis may be found in the fact that this is also the age period of maximal susceptibility to radiation-induced lymphoid tumors in mice(7), and recent studies have confirmed the suspected operation of hormonal and indirect factors in the induction process(8,9). In addition administration of estradiol benzoate(10,11) or testosterone(12) at the time of irradiation has been reported to increase the mortality rate in mice following irradiation. Further studies will be necessary to determine whether this effect is related to physiologic or direct toxic properties

---

7. Kaplan, H. S., *J. Nat. Cancer Inst.*, 1948, v9, 55.

8. Kirshbaum, A., Shapiro, J. R., and Mixer, H. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 632.

9. Kaplan, H. S., Marder, S. N., and Brown, M. B., PROC. SOC. EXP. BIOL. AND MED., to be published.

10. Treadwell, A., Gardner, W. U., and Lawrence, J. H., *Endocrinology*, 1943, v32, 161.

11. Patt, H. M., Straube, R. L., Tyree, E. B., Swift, M. N., and Smith, D. E., *Am. J. Physiol.*, 1949, v159, 269.

12. Ellinger, F., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 616.

of these substances.

The relative resistance of mice irradiated at the age of one day is not shared by mice irradiated before birth. A relatively high mortality has been observed among a small group of mice exposed to intrauterine irradiation. Mice irradiated one day before birth survive considerably longer than mice irradiated 2 or 3 days before birth. These findings suggest that profound alterations occur shortly before, during or immediately after birth which render mice less susceptible to irradiation.

Sex does not appear to affect susceptibility of mice to whole-body irradiation. A possible exception is the 90 day age group in which a suggestively greater proportion of females than males died. It is of interest that a high mortality rate (50 per cent) has been observed in a small group of pregnant female mice 90 days of age at the time of irradiation. Further investigation of the influence of pregnancy on susceptibility to whole-body irradiation is in progress.

Some of the results reported here may be helpful in the design of future experiments concerned with radiation mortality in mice (and presumably also in other species). Attention is particularly directed to the need for controlling age as a variable, and to the desirability of selecting an age such that the anticipated mortality may facilitate statistical analysis of the experimental results. The usual 30 day observation period following irradiation, which is adequate for older mice, should probably be extended to 60 days when suckling mice are being studied, and weaning time must be suitably controlled among different experimental groups.

Summary and conclusions. (1) The age at which mice are irradiated profoundly alters their resistance to a single exposure of whole body irradiation. Mortality is low after irradiation at 1 and 15 days of age, very high at 30 days, and decreases rapidly with increasing age thereafter. The possible mechanisms of this effect are discussed. (2) The response of mice to whole body irradiation is not related to body weight except insofar as weight reflects a particular age range. (3) In general, sex appears to exert no significant influence on the capacity of mice to withstand whole body irradiation.

Received March 5, 1951. P.S.E.B.M., 1951, v76.

Influence of Concentration of Thromboplastin on Prothrombin Time of Human and Dog Plasma.* (18611)

Armand J. Quick and Clara V. Hussey.

From the Department of Biochemistry, Marquette School of Medicine, Milwaukee, Wis.

In the development of the one-stage prothrombin test, it was observed that on adding increasing amounts of thromboplastin, a minimum clotting time was obtained which remained constant irrespective of the excess of thromboplastin(1). In the present study, an attempt is made to correlate quantitatively the concentration of thromboplastin required for obtaining minimal clotting values when the prothrombin activity of the plasma is varied by different experimental means.

Methods. As the source of thromboplastin rabbit brain dehydrated with acetone as previously described(2) was employed. To test the potency of the material, various concentrations were prepared by mixing weighed amounts with 5 cc physiological saline and incubating the mixture at 50°C for 20 minutes. The various concentrations of thromboplastin which ranged from 1 to 250 mg in 5 cc of saline (0.02 to 5.0%) were tested on

---

* This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1. Quick, A. J., *Am. J. Physiol.*, 1936, v114, 282.

2. Quick, A. J., *J.A.M.A.*, 1938, v110, 1658.

TABLE I. Effect of Concentration of Thromboplastin on Prothrombin Time.

Thromboplastin mg*	250	200	150	100	75	50	25	10	5	2½	1
Fresh human plasma:	Prothrombin time in seconds										
With .02 M $CaCl_2$	13	13	13	13	13	14½	17	21	24	29	38
With .01 M $CaCl_2$	12	12	12	12	12	13	16	20	22	27	36
Fresh human plasma .9 cc D.† rabbit plasma‡ 0.1 cc											
With .02 M $CaCl_2$	13	13	13	13	13	16	17	20	21	28	32
With .01 M $CaCl_2$	12½	12½	12½	12½	12½	14½	16	18	19	21	18
Stored human plasma .9 cc D.† rabbit plasma‡ 0.1 cc											
With .02 M $CaCl_2$	8	8	8½	9	9½	10	10½	13	16	17	18½
With .01 M $CaCl_2$	8½	8½	9	9	9½	10	10	13	16	17	18½
Fresh dog plasma:											
With .02 M $CaCl_2$	6	6	7	7½	8	9	11	12½	14	16	22
With .01 M $CaCl_2$	6	6	7½	8	9	10	12	13½	17	21	29
Dog plasma (Dicumarol) I§	12	12	12	12	12	13	13½	14	15	16	17½
Dog plasma (Dicumarol) II§	25	25	25	25	25	27	28½	31	36½	38½	41½
Dog plasma (vit. K deficiency)§	20	20	20	20	20	21½	23	25	27½	34	40

* Amount of thromboplastin mixed with 5 cc saline solution.

† Deprothrombinized.

‡ The prothrombin was removed with tricalcium phosphate according to the procedure of Quick and Stefanini(5).

§ Only .02 M $CaCl_2$ was used.

oxalated human and dog plasma according to the one-stage prothrombin test(2). In the collection of the blood, silicone coated glassware was employed to avoid the changes that occur when blood is in contact with glass. The thromboplastin requirement of both fresh and stored human oxalated plasma were studied. For the latter, the blood was put in glass test tubes and kept in the refrigerator at 4°C for 2 to 4 days. In testing the prothrombin activity of stored plasma, the labile factor which disappears on storage had to be replaced. This was done by adding 1 volume of deprothrombinized rabbit plasma, prepared by adsorption with tricalcium phosphate, to 9 volumes of stored plasma(3). Rabbit plasma has an exceedingly high concentration of labile factor. For a control, deprothrombinized rabbit plasma was added to fresh human plasma, and the requirement of thromboplastin compared with unmixed fresh human plasma. Likewise normal dog plasma was compared with that obtained from animals that had been treated with Dicumarol.

Results. From the data in Table I, it can be seen that the concentration of thromboplastin obtained by mixing and extracting 75 mg of dehydrated rabbit brain with 5 cc physiological saline solution, yielded maximum activity when tested on fresh human oxalated plasma. In contrast to this, stored human plasma with its labile factor restored by means of added deprothrombinized rabbit plasma, required 200 mg of thromboplastin in 5 cc of saline. Furthermore, the minimum prothrombin was 8 seconds in contrast to the 12 seconds of fresh human plasma. The addition of deprothrombinized rabbit plasma to fresh human plasma did not shorten the prothrombin time but actually prolonged it slightly, and did not alter the thromboplastin requirement.

Normal dog plasma required 200 mg of thromboplastin in 5 cc saline for a minimum prothrombin time of 6 seconds, but when the prothrombin level was reduced by Dicumarol, 75 mg was sufficient to obtain the shortest constant prothrombin time.

It was noted that 0.02 M $CaCl_2$ gave the shortest prothrombin time for all plasmas studied except fresh oxalated human plasma. For the latter, 0.02 M $CaCl_2$ had a slight but unmistakable depressing action whereas 0.01 M did not. This was consistently observed only when blood was collected and kept in silicone-coated glassware. When blood was in contact with glass this inhibitory action of calcium was lost.

---

3. Quick, A. J., and Stefanini, M., *J. Lab. and Clin. Med.*, 1948, v33, 819.

Discussion. When human oxalated plasma is stored in glass, its prothrombin time becomes prolonged because of the disappearance of an agent which we have designated labile factor and which is probably the same as Factor V of Owren, the Ac-globulin of Seegers, the accelerator by Fantl, and the thromboplastin co-factor of Honorato. On restoring the labile factor by the addition of deprothrombinized rabbit plasma, which has a high concentration of this agent, the prothrombin time becomes 8 instead of 12 seconds, the normal for fresh human plasma. This finding can be interpreted as being due either to an increase in active prothrombin or to the formation of an agent which accelerates the conversion of prothrombin to thrombin as Alexander and his associates maintain(4). If such an accelerator exists, it is not identical with the labile factor, because the addition of the latter to fresh plasma does not increase prothrombin activity.

Aged human plasma with the labile factor restored requires definitely more thromboplastin for optimum prothrombin activity than does fresh plasma. This is independent of the labile factor since an excess added to fresh plasma has no effect. Stored human plasma behaves like fresh dog plasma in respect to thromboplastin requirement. When, however, the prothrombin level of dog plasma is reduced by Dicumarol or vitamin K deficiency, less thromboplastin is needed to obtain the constant minimum prothrombin time. This suggests that the thromboplastin requirement varies with the concentration of prothrombin. If this conclusion is correct, the increase in prothrombin activity in stored human plasma may be explained by an actual increase in active prothrombin. Quick and Stefanini(5) have postulated that the prothrombin in human blood is partly in an active form and partly in a precursor state which they designated prothrombinogen.

Summary. The amount of thromboplastin required for optimum prothrombin activity increases when human plasma is stored and the lost labile factor replaced. It decreases in dog plasma when the prothrombin level is reduced by means of Dicumarol or vitamin K deficiency. It is concluded that a direct relationship exists between the concentration of prothrombin and the amount of thromboplastin, for obtaining a minimal constant prothrombin time.

---

4. Alexander, B. and Landwehr, G., *Am. J. Physiol.,* 1949, v159, 322.

5. Quick, A. J. and Stefanini, M., *J. Lab. and Clin. Med.,* 1949, v34, 1203.

Received March 9, 1951. P.S.E.B.M., 1951, v76.

Endothelial Damage by X-Rays Disclosed by Lymph Fistula Studies.*
(18612)

R. R. BIGELOW, J. FURTH, M. C. WOODS, AND R. H. STOREY.
(With the assistance of M. M. Knoohuizen and M. H. Ross)
From the Biology Division, Oak Ridge National Laboratory, Tenn.

Studies of the rate of disappearance of varied substances from the blood of rabbits and mice(1) led to the assumption that capillary permeability is greatly increased in these species by exposure to large (LD-50) doses of X-rays. Tagged plasma, homologous and heterologous erythrocytes, and other tagged large-molecular substances when injected into the blood disappeared from the blood stream of X-rayed rabbits and mice (2 to 14 days after irradiation) faster than from that of normal control animals(1,2). In order to bring direct evidence for heightened capillary permeability and to ascertain its

---

* Work performed under Contract No. W-7405-eng-26.

1. Wish, L., Furth, J., Sheppard, C. W., and Storey, R. H., *Am. J. Roentg.,* in print.

2. Furth, J., Andrews, G. A., Storey, R. H., and Wish, L., *S.M.J.,* 1951, v44, 85.

magnitude, lymph fistulas were prepared in dogs and rats and the cellular and chemical composition of the lymph was studied. In addition the rate of appearance in the lymph of intravenously injected tagged homologous plasma was assayed in both X-rayed and normal animals as will be described in the report which follows.

Technic. Lymph fistulas were prepared in dogs by a technic similar to that described by Brown and Hardenberch(3). Polyethylene† and polyvinyl‡ tubing was inserted into the thoracic duct at the left supraclavicular region and another into a large tributary of the external jugular vein. The connecting loop was exteriorized. In rats the procedure was that used by Bollman and associates(4). The dogs used were mongrels of both sexes. The rats were Wistar rats weighing approximately 240 to 370 g. By disconnecting the loops all injections and samplings could be done without further surgical intervention. Intravenous injections were made through the venous cannula. Aureomycin was given routinely on 3 consecutive days after operation. In the final phase of the work the cannulas were flushed with heparin but since this is inadequate to prevent clotting in normal dogs and exaggerates the bleeding tendency in irradiated dogs, it was abandoned.

Results. In normal dogs the lymph-venous anastomosis remained serviceable for about 2 to 3 days only, when clotting caused closure of the fistulas. In X-rayed animals the fistulas remained patent for as long as 13 days after operation. Closure of the lymph cannula was the usual cause of the cessation of flow in the anastomosis. The venous fistulas remained open or could be readily reopened. The maintenance of flow through the fistulous anastomosis in X-rayed dogs was due in part to the clotting disturbance caused by irradiation and in part to the increased lymph flow in X-rayed animals.

In normal dogs the rate of flow through the fistula was less than 1 cc per minute, while in several X-rayed dogs it exceeded 1 cc and in one dog reached 5 cc per minute. That increased flow, itself, is evidence of heightened permeability(5) was at first not appreciated by us and therefore exact flow rate determinations were not made except in the clearance studies.

The most striking finding was the presence of blood in the lymph of X-rayed rats (Table I) and dogs (Table II). In the lymph of normal rats the number of erythrocytes is a small fraction of that of lymphocytes. In X-rayed rats the lymph was grossly bloody, and this was noted before insertion of the cannula. In the normal dogs studied, the number of erythrocytes in the lymph was also much below that of lymphocytes. The slightly excessive number of erythrocytes occasionally caused by operative trauma dropped within a day. In the lymph of 6 irradiated dogs studied 3 to 17 days after exposure (two are detailed in Table II) the erythrocyte count was ten- to a hundredfold that of the lymphocyte count. At 2 days after irradiation there was little evidence of passage of erythrocytes into the lymph. Permeability as evidenced by the very high erythrocyte counts seemed to increase with time, reaching a maximum at about 7 to 14 days.

There is evidence that X-rays within the dose tolerated by a mammal do not injure erythrocytes(*cf.* 2,6). Yet, the anemia after massive irradiation is in excess of that caused by death of aging cells in absence of erythrogenesis(*cf.* 2,7). The findings here reported indicate widespread extravasation with flooding of the lymph-compartment by erythrocytes. The well-known finding of erythrophagocytosis and later massive hemosiderosis in lymph sinuses indicates that many of the extravasated erythrocytes are destroyed.

---

3. Brown, C. S., and Hardenberch, E., *Naval Medical Research Institute Project No.* NM 006 012.04.31, July 18, 1950.

† Purchased from Clay-Adams Company, New York, New York.

‡ Obtained through the courtesy of Irvington Varnish and Insulator Company, Irvington, New Jersey.

4. Bollman, J. L., Cain, J. C., and Grindlay, J. H., *J. Lab. Clin. Med.*, 1948, v33, 1349.

5. Drinker, C. K. and Yoffey, J. M., Lymphatics, Lymph, and Lymphoid Tissue, Harvard Univ. Press, Cambridge, Mass., 1941.

6. Kahn, J. B., and Furth, J., in preparation.

7. Dunlap, C. E., (Warren S.), *Arch. Path.*, 1942, v34, 562.

TABLE I. White and Red Cell Counts in Lymph and Blood of Normal and Irradiated Rats.

	Lymph		Blood	
Rats	W.B.C. × 10^3	R.B.C. × 10^3	W.B.C. × 10^3	R.B.C. × 10^6
		Normal (5)*		
Extremes	5.4-22.5	0-1.5	12.5-21.3	8.0-10.2
Avg	15.0	0.31	14.3	9.0
		X-rayed† (8)*		
Extremes	0.2- 1.5	23.5-1070.0	0.3- 3.2	4.6-10.0
Avg	0.7	523.2	1.0	7.6

* No. of animals in group.

† 6 to 11 days after 650-750 r. The factors of irradiation were 250 Kvp, 30 ma, 3 mm Al, TSD 93.7 cm, 55 r/min.

All irradiation and monitoring was done by John J. Lane.

TABLE II. White and Red Cell Counts in Lymph and Blood of Normal and Irradiated Dogs.

	Lymph		Blood	
Dogs	W.B.C. × 10^3	R.B.C. × 10^3	W.B.C. × 10^3	R.B.C. × 10^6
		Normal* (4)†		
Extremes	26.5-39.7	0.4-7.8	19.4-23.3	5.0-6.6
Avg	32.4	3.2	20.7	5.8
		Dog I		
8 days post 350 r	0.5	125.0	1.0	5.3
10 " " "	1.0	115.0	1.0	3.5
13 " " "	0.4	155.0	0.3	5.1
17 " " "	0.4	240.0	0.4	4.3
		Dog II		
2 days post 350 r	3.8	3.3	4.5	—
6 " " "	4.4	2.1	2.1	—
8 " " "	1.0	134.5	2.0	—
11 " " "	—	998.5	2.0	—
13 " " "	3.0	1060.0	0.3	—

* One or 2 days after establishment of fistula.

† No. of animals in group.

The factors of irradiation were 250 Kvp, 30 ma, 3 mm Al, TSD 11.7 cm. Proximal skin dose 49 r/min. Animal turned over at half-time exposure.

Postmortem examinations confirmed these long-known characteristic radiation changes. Every dog autopsied exhibited a hemorrhagic tendency. Red-colored lymph nodes were a common finding; the thoracic duct of several dogs dissected was red like a vein as were numerous lymphatics leading to lymph nodes.

Summary. The degree of capillary permeability was assayed in dogs and rats by studying the appearance of components of the blood in the lymph. Marked capillary permeability was indicated in dogs and rats exposed to approximately LD-50 doses of X-rays by the appearance of large numbers of erythrocytes in the lymph. The data indicate that the reduced red cell counts after irradiation are due to the flooding of the lymph compartments and tissue spaces by erythrocytes. The massive erythrophagocytosis and hemosiderosis in sinuses of lymph nodes of irradiated hosts indicate that many of the erythrocytes leaving the blood capillaries are destroyed. Loss of erythrocytes caused by capillary damage and death of aging erythrocytes with inhibition of erythrogenesis(*cf.* 2) are the main causes of radiation anemia

---

Received March 12, 1951. P.S.E.B.M., 1951, v76.

Some Effects of Paracolon Bacteremia.* (18613)

RUSSELL M. NELSON† AND CLARENCE DENNIS.

From the Department of Surgery, School of the Medical Sciences, University of Minnesota.

We and our associates described the loss of many dogs from poorly understood causes after total body perfusions with a combined pump-oxygenator apparatus(1). Many of these dogs died of a hemorrhagic complication leading to profuse gastro-intestinal hemorrhage. We previously described the production of a hemorrhagic state by the canine infusion of hemolyzed whole blood(2). This condition was characterized by gastro-intestinal hemorrhage, serous cavity effusions, disseminated petechiae, prolongation of blood coagulation time, and death. It was determined that plasma hemoglobin was not the offending agent and that other etiological factors must be operating(2).

Subsequently, it has been learned that the blood used in these and other experiments was heavily contaminated with bacteria, with a Gram-negative micro-organism, the Paracolon bacillus predominating. Colony counts were, of course, much higher in blood which had been removed from the refrigerator for several hours, as was the case with the blood that had been hemolyzed. The control, nonhemolyzed blood, which had been refrigerated almost constantly, had very little bacterial growth. With this information, then, it was decided to ascertain the effects of intravenous canine infusion of these bacteria alone, uncomplicated by the variables of simultaneous blood administration.

Methods. Dogs of 23 kg average weight were anesthetized with sterile solutions of pentobarbital given intravenously. With strict aseptic technic throughout, a small inguinal incision was made in order to facilitate the obtaining of arterial blood samples. An 18-gauge needle was inserted into the vein of the foreleg, to which was connected the intravenous infusion flask. A 14 hour culture of the paracolon bacillus *(Paracolobactrum intermedium)* was centrifuged, the supernatant broth discarded, and the small residue in the bottom of the culture tubes resuspended in 50 cc of sterile saline solution. This suspension was infused intravenously into 9 dogs. The average time for infusion was 10 minutes. Four other dogs served as controls, receiving only sterile infusion of the same volume, in addition to the prolonged anesthesia (about 6 hours).

Results. All 9 experimental dogs died. All but 2 died within 14 hours, with the shortest period of survival being 2½ hours. Prior to death, there were retching, salivation, hyperventilation, muscular weakness, frequent loose stools, which were often bloody, and coma. No such ill effects were recognized in the control dogs. All but one of the experimental dogs showed an increase in coagulation time, and all but 2 showed gross gastro-intestinal hemorrhage.

Blood cultures were taken at 4 periods: 1. pre-infusion, 2. immediately after infusion, and the others at 3 and 5 hours. All the control and pre-infusion cultures were sterile. The culture drawn immediately after the bacterial infusion exhibited extremely heavy growth. The 3 hour culture still contained a heavy growth, but at 5 hours, the bacterial count had diminished considerably, indicating that the bacteria were being removed from the circulating blood stream.

Comment. Several reports have appeared in the literature of experiments dealing with metabolic aspects of shock, and perfusions with artificial heart, lungs and kidneys performed without bacteriologic proof of asepsis.

---

* Supported by a research grant from the U.S.P.H.S. and a grant from the Graduate School of the University of Minnesota.

† Formerly Research Fellow, U.S.P.H.S., now U.S. Army Reserve Corps, Army Medical Service Graduate School, Washington, D. C.

1. Dennis, C., Karlson, K. E., Eder, W. P., Nelson, R. M., Eddy, F. D., and Sanderson, D., *Surg.*, 1951, in press.

2. Nelson, R. M., Eder, W. P., Eddy, F. D., Karlson, K. E., and Dennis, C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 208.

For instance, Frank, Seligman, and Fine(3) described a similar syndrome in dogs which had been subjected to exsanguination to shock levels, and then reinfused with blood, which had been collected in non-sterile containers, in an effort to evaluate the stage of irreversibility of shock. The similarity of this syndrome to that seen on occasion in human patients is striking(4).

Waisbren has found that patients with advanced cirrhosis not infrequently develop a hemorrhagic syndrome in association with Gram-negative spore-former bacteremia(5).

Aub and his associates reported that many of the effects of trauma to muscle in the dog are attributable to contaminating Clostridia and concluded with a plea for care in eliminating sepsis in muscle trauma experiments(6). Our present report extends the scope of the importance of asepsis and calls attention to the lethal effect of organisms previously considered non-pathogens.

Conclusion. Dogs receiving an intravenous infusion of a 14 hour culture of the Paracolon Bacillus exhibited profound illness and rapid death. Canine experiments in which conclusions are to be based on survival in apparent health for more than 2 or 3 hours should be conducted with precise aseptic technic rather than general cleanliness alone, in order to eliminate the influences of bacterial contamination.

The authors express thanks to Dr. J. T. Syverton for kindly counsel and aid in the prosecution of this study.

---

3. Frank, H. A., Seligman, A. M., and Fine, J., *J. Clin. Invest.*, 1945, v24, 435.

4. Friesen, S. R., and Nelson, R. M., *Southern Surgeon*, 1951, in press.

5. Waisbren, Burton A., personal communication.

6. Aub, J. C., Brues, A. M., Dubos, R., Kety, S. S., Nathanson, I. T., Pope, A., and Zamecnik, P. C., *War Med.*, 1944, v5, 71.

Received February 28, 1951. P.S.E.B.M., 1951, v76.

Central Nervous System Activity of Some 2-Pyridyl Compounds.* (18614)

J. F. O'LEARY, D. E. LEARY, AND I. H. SLATER. (Introduced by H. C. Hodge)

From the Divisions of Orthopaedic Surgery, of Surgery, of Pharmacology and Toxicology, of Radiation Biology, of the University of Rochester School of Medicine and Dentistry.

In studies employing our previously described technics used in screening drugs for anti-convulsant activity(1), a variety of chemical structures containing a 2-substituted pyridine grouping have been investigated (Table I). Several piperidine and quinoline compounds of similar configuration have been examined for comparative purposes. The syntheses of a large majority of these compounds have been described recently by Boekelheide and Agnello(2).† The pharmacological actions of previously studied pyridine derivatives have been reviewed by Von Oettinger(3). More recently Fromherz and Spiegelberg(4,5) have investigated a series of 3-substituted pyridine compounds from a pharmacodynamic viewpoint, and Valenzuela and Huidobro(6) have studied the effects of some similar compounds upon neuromuscular transmission. No studies of the presently considered 2-substituted pyridine compounds re-

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Slater, I. H., O'Leary, J. F., Leary, D. E., *J. Pharmacol.*, 1950, v100, 316.

2. Boekelheide, V. and Agnello, A. J., *J. Am. Chem. Soc.*, 1950, v72, 5005.

† These investigators have kindly supplied us with these compounds.

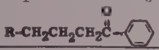
3. Von Oettingen, W. F., *The Therapeutic Agents of the Pyrrole and Pyridine Groups*, Edwards Brothers, Inc., Publishers, Ann Arbor, Mich., 1936.

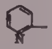
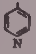
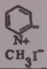
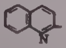
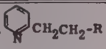
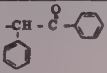
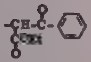
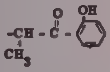
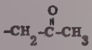
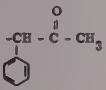
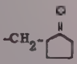
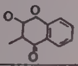
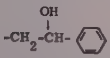
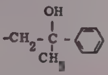
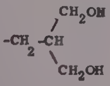
4. Fromherz, K., *Schweiz. Med. Wochensch.*, 1949, v79, 521.

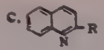
5. Fromherz, K., Spiegelberg, H., *Helv. Physiol. Acta*, 1948, v6, 42.

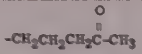
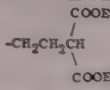
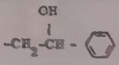
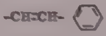
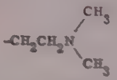
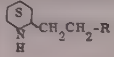
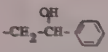
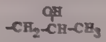
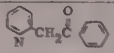
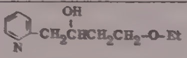
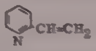
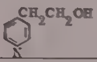
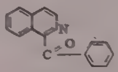
6. Valenzuela, F., and Huidobro, F., *J. Pharmacol.*, 1948, v92, 1.

TABLE I. Mean Paralyzing, Anticonvulsant, and Lethal Doses of Some Pyridyl, Piperidyl, and Quinolyl Compounds After Intraperitoneal Injection in White Mice.

A. 

Code	R. Substituent	LD_{50}	ED_{50}	PD_{e50}	$\frac{LD_{50}}{ED_{50}}$	$\frac{LD_{50}}{PD_{e50}}$
		mM/kgm	mM/kgm	mM/kgm		
EJA II		0.89 ±.06	0.27±.02	.084±.014	3.3	10.7
M-3167		1.37 ±.12	0.68±.05	0.22 ±.03	2.0	6.2
VB-25		.085±.012	None	None	-	-
170-2		0.64 ±.11	Convulse	0.21±.02	-	3.2
	B. 					
M-85		1.27 ±.19	Convulse	0.48*	-	2.6
M-96-2		2.6	2.14±.31	0.44±.17	1.2	5.9
EJA V		3.2 ±.13	1.9 ±.29	0.47	1.7	6.8
VB-19	 $-CH_2-\overset{O}{\overset{\Vert}{C}}-CH_3$	1.7	1.4	None	1.2	-
M-16-1		1.88 ±.21	0.55±.01	0.26±.15	3.4	7.2
M-5		1.8	1.5	None	1.2	-
EJA III		0.15 ±.03	None	None	-	-
M-11		1.88 ±.07	0.59 ±.03	0.24±.01	3.2	7.8
M-3066		2.31 ±.18	0.85±.09	0.25±.19	2.7	9.2
VB-15		4.8	None	None	-	-
VB-10	$-CH_2OH$	6.6	None	None	-	-
VB-11	-OH	11.4	None	None	-	-

C. 

Code	R. Substituent	LD_{50}	ED_{50}	PD_{e50}	$\frac{LD_{50}}{ED_{50}}$	$\frac{LD_{50}}{PD_{e50}}$
179-1	 $-CH_2CH_2CH_2C(=O)-CH_3$	2.30 ±.13	1.45±.09	0.71	1.6	3.6
M-105	 $-CH_2CH_2CH(COOEt)_2$	1.81 ±.30	None	None	-	-
M-86		3.1	Partial	0.46±.07	-	6.7
M-86-1		2.7	None	None	-	-
M-2-D	$-CH_2CH_2OH$	4.0	2.0	1.3	2.0	3.1
M-52-3	 $-CH_2CH_2N(CH_3)_2$	0.75 ±.05	None	None	-	-
	D. 					
M-3678		0.38 ±.03	None	None	-	-
VB-16	 $-CH_2-CH(OH)-CH_3$	0.89 ±.13	None	None	-	-
VB-12	$-CH_2OH$	1.5	None	None	-	-
	E. Other Compounds					
AKA-3		2.2 ±.19	1.5 ±.12	0.47±.06	1.2	4.7
WG-13-6		4.1 ±.63	3.2 ±.50	1.2 ±.10	0.8	2.9
VB-13		2.7	None	None	-	-
VB-14		10.8	None	None	-	-
W-43-1		1.05	None	None	-	-

* Values showing no standard errors were obtained from data having no intermediate partial responses between total response and no response, due to the extreme steepness of the regression lines of these assays. Inclusion of extra intermediate dose level groups was precluded in most cases by lack of adequate amounts of the compounds. The error of these values should not exceed ± 25%.

lating to central nervous action have been described.

Experimental. Table I shows the dose of the various compounds administered to mice required to paralyze half the animals (ED_{50}, loss of righting reflex). Also shown are the doses required to protect half the mice against the tonic phase of maximal electrical seizure pattern induced by a shock of 8 milliamperes of current applied for 0.5 second through ear electrodes. The ratios of these doses to the median lethal dose are given also for each compound. In most instances, several groups of 5 or 10 mice each were used to determine each value shown. The various dosages shown in Table I were obtained together with their standard errors where shown, by the plotting procedure of Miller and Tainter(7).

These compounds showed no significant protection against strychnine or metrazol convulsions in experiments in which 2 lethal doses of the convulsant were injected into mice intraperitoneally 5 minutes after the intraperitoneal administration of the compound to be tested. Survivals at the end of one hour were counted and a mean protecting dose, if any, was calculated.

EJA II, phenyl γ-(2-pyridyl) propyl ketone, was the most potent compound studied.

Discussion. It is noteworthy that many of these compounds exhibit potent action both as paralyzing agents and as anti-convulsants against electro-shock. However, the great majority of these compounds were characterized by a short duration of action, *viz.,* 5 to 20 minutes. Since our screening procedures measure protective activity against strychnine or metrazol for one hour, few of the compounds appeared to be effective anti-convulsants against these agents although in many cases there was a significant prolongation of survival time. Protection technics utilizing intravenous titration-to-convulsion or death(8) measure the instantaneous potency of the anticonvulsant without the immediate consideration of the probable duration of action. Such studies should provide more useful information concerning the site of action of anti-convulsant drugs having short action. These compounds have been found to be primarily central nervous depressants, although in two instances (M-85 and 170-2) stimulant activity is observed as indicated by excitement and convulsions (Table I). In mice the depressant action is characterized in most cases by an initial arching of the neck and stiffening of the limbs, with paralysis ensuing shortly thereafter. A similar action has been observed in preliminary experiments on rats, rabbits, and cats.

Because of the short duration of action of these compounds, they show little promise of clinical application at present. However, in view of their interesting central action, further studies on these and related compounds are being undertaken.

---

7. Miller, L. C., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 261.

8. Orloff, M. J., Williams, H. L., and Pfeiffer, C. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 254.

Received January 26, 1951. P.S.E.B.M., 1951, v76.

Distribution of Radioactivity in the Egg after Feeding Sodium Acetate-1-C^{14}* (18615)

DAVID KRITCHEVSKY, C. R. GRAU, B. M. TOLBERT AND BARBARA J. KRUECKEL.
(Introduced by John H. Lawrence)

From the Radiation Laboratory and the Division of Poultry Husbandry, University of California, Berkeley.

Earlier work on the distribution of radioactivity in eggs following the administration of radioactive isotopes of phosphorus, strontium and iron has been discussed in the com-

---

* This work was sponsored by the U. S. Atomic Energy Commission.

prehensive treatise of Romanoff and Romanoff (1). More recently, further work has been carried out using isotopic phosphorus(2) and calcium(3,4).

The present work was undertaken with the two-fold purpose of securing data to indicate the uptake of radioactive carbon in the egg and of obtaining from these eggs products of high enough specific activities to warrant their use in subsequent work. The isotopic carbon used was in the form of $CH_3C^{14}OONa$ and was mixed with the feed over a period of several days with a view to obtaining high activities in the eggs produced. The radioactive carbon was incorporated into the shell, albumen and yolk of every egg laid during the experimental period.

Experimental procedures. The sodium acetate-1-C^{14} (9.55 millicuries, 0.787 g, sp. act. 12.1 μc/mg) was prepared by carbonation of methyl magnesium iodide with $C^{14}O_2$ (5). The sodium acetate-1-C^{14} was dissolved in a small amount of water and the resulting solution was sprayed over the feed (a practical laying ration) which was being stirred by hand. After all the solution was added, the feed was thoroughly mixed. The labeled acetate was administered in a total of 3 feedings. The first feeding comprised 4.78 mc of acetate in 350 g of feed. Then 2.39 mc of acetate in 175 g of feed and 2.38 mc in 175 g of feed were fed on the sixth and eighth days, respectively, after the initial feeding. As soon as one portion of feed was exhausted, a new one was prepared and fed. Thus, the hen was fed labeled acetate for ten days, at which time she was sacrificed. The hen used was a Single Comb White Leghorn, maintained in a wire cage with a raised floor. The shells were washed free of adhering matter and the yolk and albumen of each egg was lyophilized separately. Each shell was freed of its shell membrane, which was lyophilized with the albumen, and then air dried and pulverized. Aliquots of the shell, yolk and albumen were converted by dry combustion to CO_2, which was collected and counted as barium carbonate. Seven eggs were collected over the 10 day period of the experiment. The eggs were collected on the second, third, fifth, sixth, eighth, ninth and tenth days after feeding was begun. The hen was sacrificed on the tenth day and the contents of the ovary were separated into 4 fractions comprising 2 large ova, a number of easily separated small ova and the ovary with the remaining ova. Each of these fractions was homogenized, lyophilized and the activity of an aliquot was determined.

Results and discussion. Romanoff and Romanoff(6) present data to show that the egg, in passing through the oviduct, spends 3 hours in the albumen secreting region and 19 hours in the uterus, where the shell is laid down. The fact that in egg 1 (which was laid within 24 hours after feeding was begun) radioactivity was present in the yolk, albumen and shell, reflects the very rapid metabolic utilization of the acetate.

In the first egg, most of the activity was found in the shell, which is understandable, since radioactive carbonate was being produced at the same time that carbonate was being utilized for shell formation. The specific activities of shells 2, 3, 5, 6 and 7 were roughly the same, the carbonate for these shells probably having been supplied by the metabolic pool. The activity of shell 4, formed soon after fresh feed was offered, was considerably higher.

The yolks exhibited a specific activity variation that parallels that of the shell, with one exception which will be noted shortly. The specific activities began to fall off as the feed was exhausted and had the hen been allowed to lay the 2 eggs represented by yolks 8 and 9, the yolk specific activities would have been 75 x 10^{-4} μc/mg and 36 x 10^{-4} μc/mg, respec-

---

1. Romanoff, A. L., and Romanoff, A. J., *The Avian Egg*, John Wiley and Sons, Inc., New York, 1949, p. 363.

2. O'Neil, J. B., Jowsey, R., Lee, C. C., Reade, M. A., and Spinks, J. W. T., *Science*, 1948, v107, 295.

3. Comar, C. L. and Driggers, J. C., *Ibid.*, 1949, v109, 282.

4. Spinks, J. W. T., Berlie, M. R., and O'Neil, J. B., *Ibid.*, 1949, v110, 332.

5. Calvin, M., *et al.*, *Isotopic Carbon*, John Wiley and Sons, Inc., New York, 1949, p. 178.

6. loc. cit., p. 223 ff.

TABLE I. Weights and Activities of Collected Eggs and Ova.*

				Shell		Albumen		Yolk	
Day	Acetate fed	Egg	Total activity of egg	Wt	Act.	Wt	Act.	Wt	Act.
1	4780								
2		1	6.4	6.50	4.8	3.35	0.2	8.56	1.4
3		2	53.0	6.22	16.9	3.27	3.8	8.61	32.3
5		3	57.3	6.95	16.1	4.40	8.3	8.81	32.9
6	2390	4	115.5	6.13	33.4	4.08	6.8	8.48	75.3
8	2380	5	56.4	6.35	20.6	4.30	11.3	8.30	24.5
9		6	132.7	6.11	13.7	3.54	8.1	8.37	110.9
10		7	129.6	5.00	10.7	4.21	5.3	9.33	113.6
—		Ovum 1	64.8	—	—	—	—	5.85	64.8
—		’’ 2	30.4	—	—	—	—	3.25	30.4
—		Ova	9.4	—	—	—	—	1.43	9.4
—		Ovary and ova	10.5	—	—	—	—	2.44	10.5

* All weights of lyophilized solid, given in g. All activities as μc.

tively, based upon average weight of yolks obtained.

Possible explanations for the low activity of yolk 5 are: (1) error in marking and perhaps in combustion of the yolk, or (2) failure of the hen to ovulate this yolk at the normal time, that is, to have produced an egg on the fourth day between eggs 2 and 3. No instances of such "held" yolks were found in the studies of Warren and Conrad(7). Further investigation into the specific activities of various yolk fractions will indicate which of these possibilities is the more valid.

The specific activity of all the albumen fractions was lower, which might be expected considering that for inclusion into albumen, the acetate fragment or its carboxyl moiety would be required to traverse longer metabolic pathways than for inclusion into shell or yolk. In general, a gradual increase of specific activity of the albumen fractions was observed, which would seem to indicate that the radioactive portion of this fraction was being supplied by the metabolic pool.

In all, 666 μc (6.96%) of the total fed activity was recovered in the eggs; of this, the yolks contained 495.5 μc (5.18%), the shells 116.2 μc (1.21%), the albumen 43.8 μc (0.46%) and the ovary and ova 10.5 μc (0.11%).

Summary. The data indicate that upon feeding of carboxyl-labeled sodium acetate, the activity is rapidly incorporated into all portions of the egg. The acetate is utilized immediately and a comparatively small percentage of radioactivity is diverted to the metabolic pool. Steady feeding of labeled material is required in order to maintain a high specific activity in all portions of the egg.

It is a pleasure to acknowledge the interest shown by Professors Melvin Calvin and Samuel Lephovsky during the course of this work.

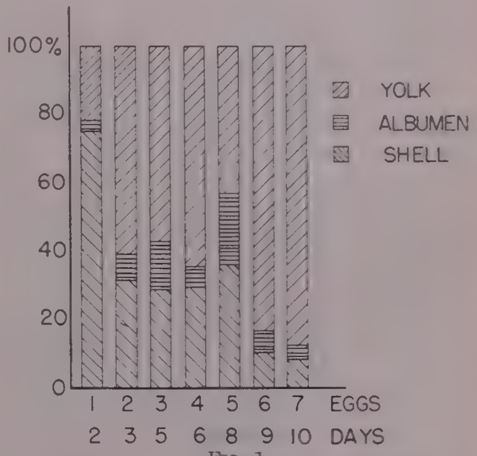


FIG. 1.
Distribution of total activity in laid eggs.

---

7. Warren, D. C. and Conrad, R. M., *J. Agr. Research*, 1939, v58, 875.

Received February 14, 1951. P.S.E.B.M., 1951, v76.

Effects of Tyrocidine on *Trypanosoma cruzi* Cultures. (18616)

Yost U. Amrein. (Introduced by Gordon H. Ball)

From the Department of Zoology, University of California at Los Angeles.

Weinman(1) reported among other findings that cultures of *T. cruzi* when subjected to certain concentrations of tyrocidine exhibited abnormal macroscopic and microscopic cultural characteristics. Instead of the thin whip-like flagellated forms prominent in normal cultures of *T. cruzi*, organisms with enlarged rounded posterior extremities were found in the presence of 5 γ/cc.

It is a question if the changes described above by Weinman were specific for tyrocidine or were the result of other factors, such as the hydrogen ion concentration of the cultures. Furthermore, it was considered important to test the effects of tyrocidine at various concentrations on the growth of the cultures themselves under controlled conditions in order to discover the actual type of effect produced on the trypanosomes.

Materials and methods. The strain of *T. cruzi* employed in these studies was obtained in July, 1947 from the National Institutes of Health, Bethesda, Md. The organisms were grown in the fluid medium with solid base developed by Chang(2,3), cotton stoppered 50 ml Erlenmeyer flasks proving to be the most convenient containers. The usual procedure was to add to 9 ml sterile Seitz-filtered culture fluid 1 ml of the experimental tyrocidine dilutions and 1 ml of trypanosome culture, adjusted with normal saline to give known inoculation densities. Tyrocidine hydrochloride was kindly supplied by Wallerstein Co., N. Y. Counts of the organisms were made with a Spencer bright line haemocytometer with improved Neubauer ruling, using the white blood count technic. Finally, thin films of the parasites were made by the standard thin blood film methods. Giemsa's stain was used after fixation and preliminary staining with Jenner's blood stain.

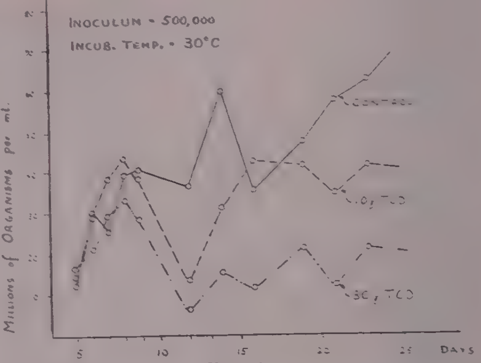


FIG. 1.

Growth curves of *T. cruzi* at 30°C in the presence of 10 γ and 30 γ tyrocidine (TCD) as compared with control cultures.

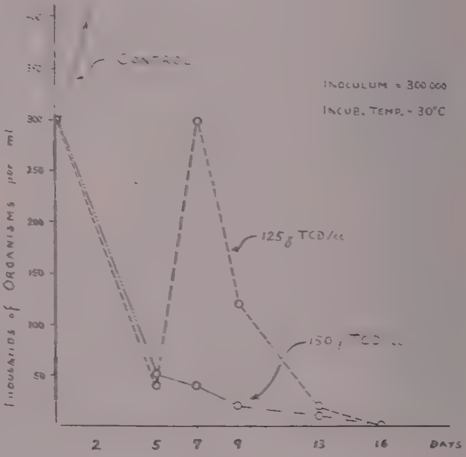


FIG. 2.

Growth curves of *T. cruzi* at 30°C in the presence of 125 γ and 150 γ tyrocidine (TCD).

Experimental results. Fig. 1 represents one of the growth curves for *T. cruzi* cultured at 30°C in the presence of 10 γ/cc and 30 γ/cc of the antibiotic. Initial density was 500000 organisms/cc. It is apparent that there is a clear-cut growth depressing effect of tyrocidine which is roughly proportional to the concentrations used. With initial inoculation densities of 40000 or 60000 organisms per ml the growth never reached sufficient density in the

---

1. Weinman, D., Proc. Soc. Exp. Biol. and Med., 1943, v54, 38.

2. Chang, S. H., *J. Inf. Dis.*, 1947, v80, 164.

3. Chang, S. H. and Negherbon, W. O., *Ibid.*, 1947, v80, 172.

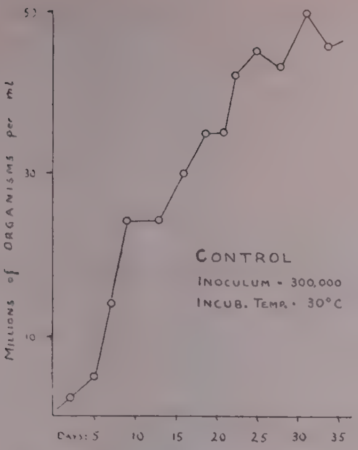


Fig. 3.

Growth curve of control culture of *T. cruzi* at 30°C drawn to different scale than Fig. 2.

control cultures to show any marked differences between these and experimental cultures.

Fig. 2 shows curves of haemoflagellate cultures grown in the presence of 125 γ/cc and 150 γ/cc tyrocidine respectively, initial density being 300000 organisms/cc. The growth depressing effect of tyrocidine is shown clearly with these concentrations of the antibiotic. Fig. 3 represents the control cultures.

Discussion. When *T. cruzi* is grown in Chang's serum-containing medium with a tyrocidine concentration above 100 γ/cc, very marked growth depression is evidenced. 200 γ/cc kills the organisms within a few hours. With 175 γ/cc living flagellates can be detected for but a day or two, while a concentration of 150 γ/cc reduces the culture to zero over a period of two weeks. In 125 γ/cc *T. cruzi* appears to show an abortive recovery peak after the initial shock of exposure to tyrocidine, yet these cultures too die out in two and a half weeks. Hotchkiss (4) has suggested that tyrocidine acts like a cationic detergent in altering the cellular membrane of bacteria; the same mechanism may be operating on the protozoan cell. Even though noticeable interference with normal growth of *T. cruzi* cultures in the presence of tyrocidine in concentrations below 100 γ/cc does occur, such inhibition can only be demonstrated clearly when relatively high initial densities of *T. cruzi* are used. If the inocula consist of less than 100000 organisms/cc, the cultures never really get a good start. Apparently the shock of subculturing to a new medium affects adversely a certain percentage of the flagellate population. Therefore, the higher the initial density, the higher the number of surviving and actively multiplying organisms.

Vacuolated and rounded leptomonad stages could first be detected on slides made after the 10th day. Usually the cultures with antibiotic substance showed this change in morphology a few days earlier than the controls. Such abnormal forms appeared when the hydrogen-ion concentration of the culture media dropped to 6 or below. This apparent close correlation between pH and morphological changes was observed both in the antibiotic-containing cultures and in the controls. These findings raise the question if the hydrogen-ion concentration *per se* is the sole causative agent for such drastic morphological changes or if these alterations are due in part to the actions of metabolites of an ageing culture.

No significant differences in growth characteristics could be observed in these experiments when inactivated instead of normal serum was used. The growth curves show that the effects of tyrocidine are only slightly influenced by temperature between 20°C and 30°C. Finally, by the time the cultures were three weeks old, no further conclusive effect of tyrocidine could be demonstrated; apparently by that time the activity of the antibiotic had practically completely vanished.

Summary. Effects of various concentrations of tyrocidine, an antibiotic substance produced by *Bacillus brevis,* have been tested on *in vitro* cultures of *Trypanosoma cruzi.* Concentrations of 100 γ tyrocidine/cc and over show a growth-depressing effect on *T. cruzi* cultures even in the presence of serum, such an effect being increased proportionately with antibiotic concentration, until with 200 γ/cc and a few hours' exposure, the lethal dosage is reached. The effects of tyrocidine *in vitro* on *T. cruzi* are only slightly influenced

---

4. Hotchkiss, R. D., *Adv. Enzymology,* 1944, v4, 153.

by temperature over a range of 20°C to 30°C. When the pH of the culture medium reaches 6.0 or lower, morphological changes in the flagellates are produced. Vacuolation and rounding up occur in both control and antibiotic-containing cultures and do not seem to be due to the influence of tyrocidine, but rather to a change in the hydrogen-ion concentration of the culture medium, with possibly the actions of metabolites in an ageing culture.

Received March 1, 1951. P.S.E.B.M., 1951, v76.

Water, Sodium and Potassium Content of Normal and Encephalomyelitic Mouse Brain.* (18617)

FRANK GOLLAN† AND MAURICE B. VISSCHER.
From the Department of Physiology, University of Minnesota.

Changes in the water and electrolyte content of brain tissue have been described in a variety of conditions. By using the method of extracellular electrolyte depletion described by Yannet and Darrow(1), Swinyard, Toman and Goodman(2) were able to show that a marked reduction occurred in electroshock and chemoshock threshold in the first 4 hours and that the threshold returned to normal in 24 hours. In a later study from the same laboratory(3) this phenomenon was further investigated and it could be demonstrated that a small but not significant increase in total brain water occurred concomitantly with a decrease in electroshock seizure threshold and that an increase in extracellular sodium and chloride concentration of cerebral cortical tissue was more closely correlated with the electroshock seizure threshold than with other factors examined. In micro-incinerated preparations and by employing a microcrystallographic method(4), changes of local distribution of potassium and sodium in the cerebral cortex in relation to experimental convulsions could be detected. It was further shown that these neuron changes involved a loss of potassium and a gain in sodium. These changes were found to be consistent in animals given metrazol or electroshock convulsions, but were absent in asphyxia, exercise, ether anesthesia and did not depend on cortical topography. In experimental cerebral concussion in the cat an increase in the volume of the brain up to 5.5% could be found(5). In dogs, however, there was no indication of swelling and there was no shifting of water from the extracellular to the intracellular phase or vice versa as determined by the water, chloride, sodium, potassium, calcium, magnesium and total nitrogen content of cerebral tissue(6). Furthermore, the ionic environment has a pronounced influence on brain tissue respiration and it could be shown(7) that potassium caused an increase in aerobic glycolysis and inhibited anaerobic glycolysis, whereas sodium lacked these effects(8).

In view of clinical data suggesting that salt depletion and hemoconcentration may be predisposing factors to the invasion of the poliomyelitis virus(9) and the low serum potassium levels found in patients with severe infantile paralysis(10), studies on the water and electrolyte content of brain tissue of mice infected by the intraperitoneal route with a

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

† Present address, Samuel S. Fels Research Institute, Yellow Springs, O.

1. Yannet, H. and Darrow, D. C., *J. Biol. Chem.*, 1940, v134, 721.

2. Swinyard, E. A., Toman, J. E. P., and Goodman, L. S., *J. Neurophysiol.*, 1946, v9, 47.

3. Swinyard, E. A., *Am. J. Physiol.*, 1949, v156, 163.

4. Colfer, H. F. and Essex, H., *Am. J. Physiol.*, 1947, v150, 27.

5. White, J. C., Brooks, J. R., Goldthwaite, J. C., and Adams, R. D., *Ann. Surg.*, 1943, v118, 619.

6. Eichelberger, L., Kollros, J. J., and Walker, E., *Am. J. Physiol.*, 1949, v156, 129.

7. Ashford, C. A. and Dixon, K. C., *Biochem. J.*, 1935, v29, 157.

8. Dixon, K. C., *J. Physiol.*, 1949, v110, 87.

9. Rinehart, J. F., *J. Ment. Nerv. Dis.*, 1944, v99, 822.

TABLE I. Composition of Mouse Brain. (Mean value, standard deviation, and probability).

	No. of animals	Wet wt, mg	Dry wt, mg	Dry wt, mg/g wet wt	Water, mg/g of wet wt	Na, mg/g of wet wt	K, mg/g of wet wt
Normal mouse brain	23	419.3 ±24.3	91.5 ±4.7	218.6 ±11.7	781.4 ±11.7	1.0 ±0.1	3.7 ±0.4
Infected mouse brain	28	430.8 ±31.4	89.9 ±6.4	208.9 ± 8.4	791.1 ± 8.4	1.3 ±0.2	3.6 ±0.5
P values for difference					.02	.01	.40

neurotropic virus seemed to be desirable.

Experimental. Four- to 6-weeks-old mice of the ZBC strain‡ were inoculated intraperitoneally with a 10^{-5} dilution of the MM encephalomyelitis virus.§ When the first signs and symptoms of the disease developed the mice were decapitated and the wet and dry weight of the whole brain were determined in a platinum crucible. The dry tissue was then moistened with 4 N sulfuric acid and ashed at 500°C for 15 hours. The ash was dissolved in 5 cc of distilled water and analyzed for sodium and potassium by means of a Perkin-Elmer flame photometer. The brains of normal mice of the same strain and age served as controls. Table I shows the results obtained in 23 normal and in 28 infected brains.

Discussion. The data show that the invasion of brain tissue with the MM encephalomyelitis virus is accompanied by significant changes in the water and electrolyte content. Of all the factors studied the most marked changes are the increase in the content of water and of sodium. These results might be interpreted in either of two ways. There might be either an accumulation of extracellular fluid containing sodium, or an increase in intracellular sodium and water. There is evidence that some of the sodium in brain tissue is intracellular(11). The proportions might change in disease, however the more simple interpretation of our findings would be the first mentioned, namely that extracellular edema occurs. Encephalomyelitic viruses affect selectively certain centers of the brain stem and do not produce uniform damage. Therefore shifts of water or electrolytes in the diseased portions may have been masked by analysing the whole brain. Such an assumption can be supported by the finding of mutual replacability of neuronal potassium and sodium in the axoplasm of the giant squid(12). It has previously been postulated that edema of the cord may contribute to the pathogenesis of the paralysis in poliomyelitis (13) and the presence of edema in the central nervous system in poliomyelitis has been described(14). Recently therapeutic measures for severe cases of poliomyelitis, like repeated blood plasma transfusions, have been based on this assumption(15,16).

Summary. 1. The infection of mice with the MM encephalomyelitis virus causes a significant increase in the water and sodium content of the brain, as compared with normal controls. 2. The potassium content of the infected brain shows a slight decrease which is not statistically significant. 3. A plausible but not conclusively demonstrated interpretation of the results is that an extracellular edema of the brain occurs with MM encephalomyelitis virus in mice.

---

‡ From the colony of Dr. John J. Bittner, University of Minn.

§ Obtained from Dr. Raymond Bieter, University of Minn.

10. Earle, A. M., *J. Ped.*, 1950, v36, 715.

11. Eichelberger, L. and Richter, R. B., *J. Biol. Chem.*, 1944, v154, 21.

12. Steinbach, H. B., and Spiegelman, S., *J. Cell. and Comp. Physiol.*, 1943, v22, 187.

13. Wickman, I., *Nerv. and Mental Dis. Monograph Series* 1913 No. 16.

14. Courville, C. B., *Pathology of the Central Nervous System,* p. 234, 2d ed., Pacific Press, Cal., 1945.

15. Eaton, R. M., and Bower, A. G., *Science*, 1949, v110, 428.

16. Bower, A. G., Eaton, R. M., Chudnoff, J. S., Affeldt, J. E., and Chaney, A. L., *Am. J. Med. Sc.*, 1950, v220, 46.

Received March 5, 1951. P.S.E.B.M., 1951, v76.

Reversal of Anabolic Effect of Testosterone. (18618)

H. Spencer Hoyt, Toland Doud, and Robert S. Hoyt.
(Introduced by Herbert M. Evans)

From the Division of Urology, Stanford University School of Medicine, San Francisco and the Department of Clinical Laboratories, Monterey Hospital, Monterey, Calif.

The anabolic effect of male hormone is well known. Nitrogen is retained and body tissue formed, with increase in body and organ weight. Whether or not this action of the male hormone is mediated through the pituitary has been studied by Simpson, Marx, Becks and Evans(1), and in a subsequent contribution by Gordon, Evans and Simpson (2) and recently by Kochakian(3). The relation between the male hormone and the growth hormone as regards their anabolic effect has been studied(4). It has been shown that there is a summation of their anabolic effects in certain aspects, with no evidence of inhibition in any effects studied.

Long-continued administration of testosterone in our hands has, however, resulted in a reversal of the anabolic effect as shown by growth retardation of the entire body. There was selective retardation in development of the organs of internal secretion. The liver and kidneys were retarded equally and slightly less than the total body weight ratio. The thyroid was selectively inhibited, and the gonad almost totally atrophied. The spleen was retarded to a greater degree than the liver and kidneys. Bone development was retarded, both in maturation, *i.e.* appearance of and development of centers of ossification, and in growth.

Method. Baby chicks 3 days old, without regard to sex, were divided into 2 groups. Four animals were given testosterone, 5 mg daily, dropped on the comb(5) from a hypodermic needle, for 77 days. Four animals received no hormone. Systemic effect of the hormone was obvious in marked growth of comb and head ornaments, with increased plumage. Body and organ weights are shown in Table I.

TABLE I.

	Male hormone for 77 days	Control	Ratio of wt Testosterone treated / Control
Total body wt (g)	450	835	.54
Organ wt as g/100 g body wt			
Liver	2.4	3.8	.63
Spleen	.082	.27	.30
Kidneys	.70	1.07	.65
Thyroid r	.03	.15	.20
Thyroid l	.06	.23	.26
Gonad	.006	.10	.06
Adrenals*	.018	.008	2.2

* Due to the plexus of veins about the adrenals and their small size, the conclusion that there was an actual AC hypertrophy is only tentative.

Note: Since writing the above, Kochakian, Chas. D., Robertson, Evangeline, and Bartlett, Mary N. have published a further report (*Am. J. Physiol.*, 1950, v163, 332.). These studies carry out the administration of testosterone for long periods, comparable to our experiments, and they describe a "wearing off" effect. We used somewhat larger doses and have shown what we interpret as an actual reversal of the anabolic effect of testosterone propionate.

We wish to thank Dr. Herbert M. Evans and Dr. Gilbert S. Gordan for helpful criticism. We also wish to thank the Ciba Pharmaceutical Co. for a generous supply of testosterone propionate.

---

1. Simpson, M. E., Marx, W., Becks, H. and Evans, H. M., *Endocrinology,* 1944, v35, 309.

2. Gordan, G. S., Evans, H. M., and Simpson, M. E., *Endocrinology,* 1947, v40, 375.

3. Kochakian, Chas. D., *Am. J. Physiol.,* 1950, v160, 66.

4. Kochakian, Chas. D., *Am. J. Physiol.,* 1948, v155-2, 255.

5. Dorfman, Ralph I., *Endocrinology,* 1948, v42, 1.

Received March 7, 1951. P.S.E.B.M., 1951, v76.

Enhancement of Oncolytic Effect of Russian Encephalitis Virus.* (18619)

ALICE E. MOORE. (Introduced by C. P. Rhoads)

From the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York City.

In the study of the effect of neurotropic viruses on transplantable tumors in mice, it was found that the oncolytic action of these viruses was so closely associated with the neurotropism that the tumor bearing animals always died of the infection(1,2). If animals were immunized either actively or passively against the viruses no tumor destruction took place(3). It seemed possible that by continuous passage from tumor to tumor in cancer bearing animals, some change might be brought about in the tropism of the virus. That tumor destruction is not inevitably associated with nervous system injury was shown by Sharpless, Davies and Cox(4). They reported that both Russian and West Nile encephalitis viruses could cause the regression of the chicken tumor RPL-12 with the survival of the host.

For the past two years we have attempted to separate the neurotropic portion of the Russian encephalitis virus from its oncolytic portion. Although the objective has not been attained, certain changes have occurred in the affinity of the virus for tumor tissue. By continuous passage it can be shown that its oncolytic ability can be enhanced. As yet no marked change has occurred in its neurotropism.

Materials and methods. The methods used in trying to produce variant strains of virus from the parent or "stock virus" have been as follows: (1) Sarcoma 180 passages. Three types of passages are reported in this study. (a) Bi-weekly passage. Groups of 3 to 5 animals were implanted subcutaneously with tumors. When the growths were approximately 10 mm in diameter, 0.05 cc of "stock virus" was inoculated directly into the neoplasm. Three or 4 days later one animal from the group was killed, the tumor removed aseptically, and a 10% emulsion made in diluent. After centrifugation, 0.05 cc of the supernatant was inoculated into the tumors of another group of sarcoma 180 bearing animals. The remaining animals of each inoculated group were allowed to die of the infection and a record kept of their day of death and the condition of their tumor. (b) One- to 2-day passage. After 96 bi-weekly passages, another series was begun. The transfers were carried out in the same way except that inoculated tumors were removed and passed at one- and 2-day intervals. An attempt at daily tumor passage was abandoned because the virus was too readily lost. (c) Passage at higher dilutions. Numerous attempts have been made to make tumor to tumor passages after the virus has been diluted. A passage began with virus from the 117th bi-weekly passage diluted to 10^{-3} at the time of each passage had undergone 16 consecutive passages before testing. Continuous passage at higher levels (10^{-5}, 10^{-7}, 10^{-8}) have been difficult to maintain because of the possibility of diluting out the virus. (2) Brain Passage. The original "stock virus" was carried by transfer from brain to brain in order to have a comparison with the tumor to tumor passage material. 0.03 cc of 10% "stock virus" was inoculated intracerebrally and serial passages at that dilution were made at bi-weekly intervals. (3) Wagnar osteogenic sarcoma passage. Continuous passage was begun by inoculation of 0.1 cc of the "stock virus" directly into palpable Wagnar tumors. At intervals of 3 or 4 days, a tumor was removed aseptically, a 10% suspension made and 0.1 cc of centrifugate inoculated into the same type of neoplasm. *Virus.* The virus of Russian Far East encephalitis was used throughout these experiments.§ Whole mouse brains were kept frozen at –40°C in the dry ice chest. They were prepared from a single stock of brains so that all virus used and referred to as "stock virus," were at the same level of passage. All dilutions were made in 10% inactivated horse serum in physiological saline. *Titrations.* All titrations for virus content of brain and tumor were made with white mice of 18-22 g in weight obtained from commercial breeders. For intracerebral titrations 0.03 cc amounts were employed, and for the subcutaneous and intraperitoneal titrations 0.05 cc was inoculated. *Detection of changes in the virus.* Although virus variation is a recognized phenomenon the detection of variants, especially if present in small amounts, is very difficult. In the studies here reported if a "pure strain" oncolytic variant without effect on normal tissue appeared, it would be possible to detect it only by its activity against a transplantable tumor since intracerebral inoculation would produce no disease. It was necessary, therefore, to set up a standard procedure which would detect any increase in the oncolytic activity of the virus. The test for changes in activity of the sarcoma 180 passage viruses and the brain passage virus was done as follows: Mice bearing sarcoma 180 implanted 4 days previously, were inoculated intraperitoneally with 0.05 cc of a 10^{-3} dilution of virus. The inoculated virus was always titered intracerebrally and usually intraperitoneally and subcutaneously. The day following inoculation tumor and brain were removed and the amount of virus contained in each was determined by intracerebral titration. At the same time 2 mm cube pieces of tumor were implanted into virus immune but tumor susceptible mice to determine its viability (bioassay). The same procedure was done 3 days and occasionally 7 days after the original inoculation of virus.

---

* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

1. Moore, A. E., *Cancer,* 1949, v2, 525.

2. Koprowski, H., and Norton, T. W., *Cancer,* 1950, v3, 874.

3. Moore, A. E., and O'Connor, S., *Cancer,* 1950, v3, 886.

4. Sharpless, G., Davies, M., and Cox, H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 270.

The time at which the sarcoma 180 is destroyed following intraperitoneal inoculation with "stock virus" at different dilutions has been determined. It is possible therefore to detect, by the method described, any increase or decrease in the oncolytic ability of the virus consequent to a procedure designed to modify that ability.

The same type of test was used for the detection of changes in the tumor effect of the Wagnar passage virus. After a number of serial passages, the virus was tested against the Wagnar tumor by inoculation of 0.05 cc of a 10^{-1} suspension of virus intraperitoneally. At the same time the "stock virus" was inoculated in the same dilution into a comparable group of tumor bearing animals. Previous experiments† had shown that in the dilutions inoculated this virus has been ineffective in causing tumor destruction. When the animals became paralyzed, usually 6 or 7 days after the inoculation, 2 or 3 from each group were killed and the viability of the tumors determined by implantation into virus immune animals. Titrations of brain and tumor were carried out at the same time. *Tissue culture.*‡ The results obtained in animals with the sarcoma 180 passage and the brain to brain passage were confirmed by tissue cul-

Per cent of Sarcoma 180 destroyed at one and three days following intraperitoneal inoculation of 0.05 c.c. of 10^{-3} dilution of Tumor to Tumor and Brain to Brain Passage.

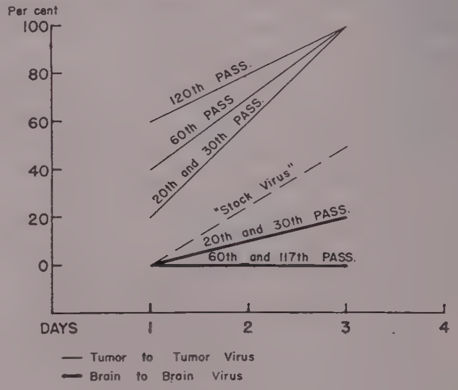


FIG. 1

---

§ Received through the courtesy of Dr. J. Casals of Rockefeller Institute.

† To be reported.

‡ The author wishes to thank Dr. C. Friend who performed these experiments.

TABLE I. Effect of Intraperitoneal Inoculation of Brain to Brain and Tumor to Tumor Passages on the Viability of Sarcoma 180.

		Titer of inoculum			1 day after inoculation			3 days after inoculation		
Passage No.		i.c.	i.p.	subq.	Tumor[*]	Brain[*]	% tumor destroyed	Tumor	Brain	% tumor destroyed
Original "stock"		9.5[§]	7.5	7.5	4.5	1.5	0	9.0	4.0	50
B to B[†]	10th	>9.0	8.0	7.5	>5.0	3.0	20	8.5	4.5	20
T to T[‡]	10	9.5	7.5	6.0	3.5	1.0	0	8.25	3.5	60
B to B	20	8.5	—	—	2.0	4.0	0	8.0	3.5	20
T to T	20	8.0	—	—	6.5	1.5	20	8.0	>3.0	100
B to B	30	9.5	—	—	2.0	<1.0	0	8.0	3.5	20
T to T	30	8.0	—	—	4.5	3.0	20	8.0	4.0	100
B to B	40	9.5	—	—	<1.0	4.0	0	7.5	2.0	0
T to T	40	7.75	—	—	<1.0	2.5	20	8.0	4.0	60
B to B	60	8.0	—	—	4.0	<1.0	20	8.0	2.0	0
T to T	60	7.5	—	—	>5.0	1.75	40	6.5	2.0	100
B to B	77	9.5	6.5	7.5	4.0	<1.0	0	7.5	2.0	60
T to T	77	7.5	5.5	6.0	4.0	<1.0	20	8.0	4.0	100
B to B	100	8.0	6.0	4.0	2.5	<1.0	0	7.5	2.5	0
T to T	97	7.5	6.5	4.5	4.0	<1.0	75	8.0	2.0	100
B to B	111	7.5	5.5	5.5	1.5	<1.0	0	6.5	1.5	0
T to T	108	8.5	6.5	7.5	6.0	1.75	25	8.5	4.0	100
1 to 2 day										
T to T	60	7.5	4.0	4.0	4.5	1.25	40	>7.0	4.0	100
B to B	117	8.5	6.5	5.5	>7.0	>3.0	0	>7.0	2.5	0
T to T	120	8.0	6.0	6.0	>7.0	3.0	60	>7.0	4.0	100
1 to 2 day										
T to T	84	6.0	6.5	5.5	4.0	>3.0	60	7.0	>5.0	100

[*] Amt of virus determined by intracerebral titration.

[†] B to B indicates brain to brain.

[‡] T to T indicates tumor to tumor.

[§] All figures represent the reciprocal of the logarithm of the LD_{50}.

ture technics. One flask containing a simple Maitland type medium consisting of 4.5 cc Tyrode's solution, 0.5 cc normal horse serum and minced tumor was inoculated with 0.5 cc brain passage virus at a dilution of 10^{-3}. A comparable flask was inoculated with the tumor passage virus. Twenty-four, 48 and 72 hours later 10 tumor pieces along with tumor from a control flask were removed and implanted into immunized mice to determine their viability. Titrations of the supernatant were also carried out.

Results. Sarcoma 180 *passage compared with brain passage.* After 20 passages it was apparent that the bi-weekly tumor to tumor passage virus was destroying the tumor to a greater extent than the brain to brain passage, and further passage has resulted in an increase in the oncolytic effect of this virus while the brain passage has lost its ability to attack the tumor in the 3 day period. In fact bioassay experiments with the 128th brain to brain passage showed that only 30% of the sarcoma 180 was destroyed 7 days after inoculation, whereas inoculation of "stock virus" invariably destroys 100% of the tumor in the same length of time. The results are shown graphically in Fig. 1. Table I gives the detailed information obtained from the bioassay experiments. It can be seen that in each test but one, the inoculated brain to brain passage virus had a higher intracerebral titer than the tumor to tumor passage so that the tumor destructive ability of the latter virus can not be due to an increase in the number of infective virus particles. The titers of brain and tumor from animals killed one and 3 days after inoculation show no great consistency. In general the tumor passage virus appears in larger amounts in the tumor than does brain passage virus (5 of 8 instances) one day after inoculation, whereas 3 days after inoculation both passages are more apt to be present in nearly equal titer (5 of 8 instances). Obviously the intracerebral titer has little bearing on whether a tumor is destroyed since tumors of animals inoculated 3 days previously with brain to brain

TABLE II. Effect of Inoculation of Brain to Brain Passage Virus and Tumor to Tumor Passage Virus on the Viability of Sarcoma 180 in Tissue Culture.

		24 hr*		48 hr*		72 hr*		96 hr*	
	Titer of virus inoc.	Titer	% tumor destruction	Titer	% tumor destruction	Titer	% tumor destruction	Titer	% tumor destruction
48th B to B†	8.0	3.0	0	6.5	60	6.0	70	7.0	87
48th T to T‡	8.0	1.5	33	5.0	50	6.0	70	7.0	100
107th B to B	9.5	5.0	50	3.0	20	2.5	25	—	—
107th T to T	8.5	3.5	100	5.0	100	1.0	100	—	—
1-2 day 52nd T to T	6.5	3.0	62	3.0	80	71.0	100	—	—

* Incubated at 37°C. Titrations and bioassay carried out at 24, 48, 72, and 96 hr.

† B to B indicates brain to brain.

‡ T to T indicates tumor to tumor.

Control tumor pieces exposed for the same length of time in the same medium grew in 100% of the instances.

passage have high titers and yet the virus has no ability to destroy the tumor tissue.

If the neurotropism of the tumor to tumor passage virus were decreased one would expect to get some survivors among the passage mice. This has happened in scattered instances but the total number is not very impressive—10 in 349 animals injected. However, when one realizes that 0.05 cc of a 10% suspension of virus is inoculated (approximately 1,000,000,000 LD_{50}'s) any survivors at all have special interest. None were observed in the brain to brain passage. The animals which survived with regressions of tumor were immune to challenge by fully virulent virus and, in most instances, reimplantation of sarcoma 180 resulted in failure of the tumor to grow. The results of testing 2 of the one to 2 day sarcoma 180 passages are shown in the last 2 experiments in Table I. So far these appear to be of the same order as the biweekly tumor passage and about the same number of survivors (14 out of 438) have been obtained. It was hoped that in this manner the particles of virus having strong tumor affinities could be selectively passed. One test of the 10^{-3} passage after 16 passages showed it to be similar to the other tumor to tumor passages.

Tissue culture experiments. Effective destruction of tumor by the Russian virus can also take place in tissue culture. Therefore the tumor to tumor passage and the brain to brain passages were tested by this means to see if the differences observed in animals could be confirmed. The set up of the test is given under *Methods*. The results obtained are given in Table II. The *in vitro* experiments showed no great difference between the brain to brain and tumor to tumor passages on the growth of the sarcoma 180 at the level of the 48th passage. However the 107th passage showed a difference between them. In this instance the tumor to tumor passages tested showed a much greater ability to destroy the tumor than did the brain to brain passage whose ability in this direction appeared to have decreased.

Wagnar passage virus compared to "stock virus." At regular intervals during serial passage, the virus was tested by bioassay experiments in the manner previously described to ascertain if it had acquired the ability to cause tumor destruction. "Stock virus" was tested in exactly the same manner each time for comparison. A summary of these experiments is given in Table III. It is clear that the Russian encephalitis virus, by continuous growth in a non-susceptible tumor, has been changed so that it is now capable of causing its destruction.

Discussion. From the results presented two conclusions can be drawn: (1) the oncolytic activity of the virus of Russian encephalitis for a susceptible tumor can be increased or decreased quantitatively by suitable procedures, (2) the activity can be modified, qualitatively so that it will cause destruc-

TABLE III. Effect of Intraperitoneal Inoculation of Wagnar Passage Virus on Viability of Wagnar Tumor Compared to the Parent Strain.

	Titration			Titer		
Passage No.	i.c.*	i.p.†	subq.‡	Tumor	Brain	% tumor destroyed
''Stock virus''	9.75§	7.5	6.5	6.5	8.0	0
41st passage	7.5 §	6.0	3.5	6.0	6.0	20
''Stock virus''	8.5	6.0	6.0	8.0	7.5	8
55th passage	9.0	7.5	3.5	8.0	8.5	64
''Stock virus''	8.5	6.0	6.5	6.5	8.0	0
79th passage	9.5	6.0	7.5	8.5	8.5	100
''Stock virus''	10.0	8.0	3.0	6.5	8.0	0
90th passage	10.0	6.5	3.0	7.5	8.5	100

* Intracerebral.

† Intraperitoneal.

‡ Subcutaneous.

§ Expressed as the reciprocal of the logarithm of the LD_{50}.

tion of a cytologic type of tumor which was originally resistant to the parent virus. These results were obtained by continuous passage of the virus in tumor or in brain tissue for relatively long periods of time. The changes in viral activity which occurred with passage were gradual. They show little indication of a discontinuous variation (mutation). Rather the product viruses can be looked upon as adaptive variants of the parent strain. They have retained their ability to attack nervous tissue and have changed only in their destructive capacity for tumor.

Viruses are well known for their capacity to vary with respect to the type of tissue which they can invade and injure. An understanding of the mechanism by which this takes place awaits further work on virus-host relationships but we can speculate on some of the possibilities, with reference to the present results with the strains.

If one thinks of a virus population as containing a number of variant particles, and there appears to be much evidence for this concept(5), it is conceivable that the above results were the result of selection. According to this interpretation the particles responsible for the neurotropism of the virus would have remained constant in number while the ones responsible for the tumor effect would have increased or decreased.

Another possibility resides in the make-up of the virus itself. At one point virus reproduces itself within the tumor without injury to its host cells. After long passage in that tissue the ability to destroy it is acquired by the virus. The transition is a gradual one. The process is reminiscent of the acquisition, by other forms, of the capacity to metabolize substrates after many generations of contact with them(6). It is conceivable that there exist in certain neoplastic cells specific components required for the anabolic processes of their reproduction. These may well be of the nature of nucleic acid precursors(7), and may be similar to those required by viruses(8). By continuous growth in a medium containing these components the virus may achieve the capacity for metabolizing one of them which is essential for the growth of the tumor cell. In this fashion a minor adaptive variation could endow a virus with oncolytic properties.

Still another possible explanation for the observed results may be offered. It has been shown that variant viruses may have different rates of growth(5) and it may be that the tumor trophic viruses become more rapidly adsorbed to the susceptible cell or that they may multiply more rapidly within the cell.

Summary. By continuous passage in the transplantable mouse tumor sarcoma 180 it has been possible to increase the ability of the virus of Russian encephalitis to destroy

---

5. Ginsberg, H., and Horsfall, F., *J. Exp. Med.*, 1949, v90, 393.

6. Ephrussi, B., *Harvey Lecture,* October 26, 1950.

7. Bendich, A., Furst, S. S., and Brown, G. B., *J. Biol. Chem.*, 1950, v185, 423.

8. Evans, E. A. Jr., *Bact. Rev.*, 1950, v14, 210.

this tumor. Conversely, continuous passage in the mouse brain resulted in a loss of oncolytic activity. In other experiments the virus of Russian encephalitis was changed by continuous passage in the Wagnar osteogenic sarcoma so that it caused destruction of this hitherto resistant tumor.

Received March 8, 1951. P.S.E.B.M., 1951, v76.

Delay of the Early Inflammatory Response by Cortisone.* (18620)

MAX MICHAEL, JR. AND C. MERRILL WHORTON. (Introduced by P. B. Beeson)

From the Lawson VA Hospital, Chamblee, Ga., and the Departments of Medicine and Pathology, Emory University School of Medicine, Atlanta, Ga.

Experimental and clinical observations(1) have shown that cortisone and ACTH alter the course of bacterial infections, usually having an adverse effect on the host. The inhibitory effect of these substances on the development of granulation tissue(2) has suggested to some that disruption of this phase of tissue reaction might explain these observations. However, since granulation tissue containing its new blood vessels, fibroblasts, and ground substance develops late in the sequence of body defense against infection, it was thought advisable to explore the effects of cortisone on the earliest tissue phases of the acute inflammatory reaction. Croton oil was used as an irritant to incite a cutaneous inflammatory reaction similar to that produced by many bacteria.

Materials and methods. White New Zealand rabbits weighing 2 kg were used. The skin of the abdomen was prepared with finetooth, animal clippers 24 hours before the experiment was commenced. Cortisone was given in single daily doses of 12.5 mg intramuscularly, beginning 24 hours before the inflammation was produced. Two drops of croton oil were placed on each of 6 separate sites on the shaved skin of the abdomen. Four longitudinal and 4 horizontal scratch marks, each approximately 2 cm in length, were made with a cutting needle through the oil; the scarified area was then rubbed with a glass rod. Paired biopsies of control and of cortisone-treated animals were made at frequent intervals under intravenous nembutal anesthesia. The sections were fixed in Zenker's solution and stained with phloxine methylene blue. The observations reported here are based on four lesions on each of 16 animals, 8 of which received cortisone.

Results. Because of the difficulty in producing uniform trauma at each site of scarification, the degree of the inflammatory response in the multiple areas of trauma of the same animal was slightly variable. There was also slight variation in response in different animals similarly treated. However, the inflammatory reaction in animals given cortisone was significantly and uniformly less intense than that in the controls.

Gross changes. In animals not given cortisone, the lesions became faintly erythematous within 20 minutes after application of the trauma. By 4 hours these had assumed an intense purplish coloration. Edema was marked by 8 hours. By 24 hours necrosis was obvious, and eschar formation had begun. On the other hand, in the animals given cortisone, the erythema could not be detected until at least 2 hours following scarification through croton oil. In these animals, the purplish discoloration appeared only after 12 to 18 hours and then was confined to the scratch marks. Edema was less than in the controls,

---

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Michael, Max, Jr., *South. Med. J.*, in press.

2. Ragan, C., Howes, E. L., Plotz, C. M., Meyer, K., and Blunt, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 718.

TABLE I. Graphic Representation of Tissue Sequences in Both Groups of Animals. Intensity of each reaction is graded from 0 to 4+, 4+ being of greatest severity. Each grading represents the average of multiple sections from each biopsy site in at least 2 animals.

			Time after stimulus										
			Minutes					Hours					
			5	10	20	30	45	1	2	4	8	12	24
Capillary dilatation		Control	2+	3+	4+	4+	4+	4+	4+	4+	4+		
		"E"	+	2+	3+	3+	3+	3+	3+	4+	4+	4+	4+
Leukocytes	Margination	Control		+	2+	3+	4+	4+	3+	2+	+		
		"E"			+	2+	2+	2+	2+	2+	+	+	+
	Migration	Control					+	+	3+	4+	+		
		"E"								+	2+	2+	+
	Infiltration of corium	Control							+	2+	3+	4+	4+
		"E"									2+	3+	3+
Edema		Control						±	+	2+	3+	4+	4+
		"E"								+	2+	3+	3+
Necrosis		Control									2+	2+	4+
		"E"									+	+	2+
Fibrin		Control									2+	2+	2+
		"E"									+	+	+

and little necrosis appeared.

Microscopically, all of the histologic components of the inflammatory reaction were delayed in rabbits treated with cortisone (Table I). Some aspects of inflammation, such as margination of leukocytes in blood vessels, were never as striking as those seen in the control animals. At 24 hours in the control animals many mononuclear cells, primarily macrophages and mast cells, were seen in addition to numerous polymorphonuclear leukocytes. Only a very few mononuclear cells were seen in cortisone-treated animals at 24 hours (Fig. 1).

The course of the inflammatory reaction and subsequent granulation tissue and healing corresponded closely to that previously described(3).

Discussion. From these observations, it would appear that cortisone has altered the inflammation provoked by a combination of croton oil and trauma in the following manner. The earliest changes of margination of leukocytes, migration of leukocytes, and formation of edema and fibrin are strikingly delayed and quantitatively reduced. Furthermore, the ultimate development of tissue necrosis was diminished in the cortisone-treated animals as compared with the controls. Dougherty and Schneebeli(4) report analogous results in studies of an allergic inflammatory reaction in mice using a tissue spread technic. They found that the percentage of polymorphonuclear leukocytes in all preparations, taken from 1 to 24 hours, was significantly lower in cortisone-treated mice when compared with controls. Moreover, the total number of cells was apparently reduced in the cortisone-treated group. Where cellular defenses are important early in the course of an infection, it is believed that this delay in the earliest inflammatory response might be partly responsible for the spreading of certain bacterial infections noted under the influence of cortisone and ACTH.

Summary. The effect of cortisone on the earliest tissue phases of the inflammatory reaction in the skin of rabbits was studied. A combination of croton oil and scarification was used as the inflammatory stimulus. In animals given cortisone, all of the tissue components of the inflammatory reaction were delayed, and some were quantitatively reduced. Vascular margination of leukocytes, migration of leukocytes from blood vessels, and the formation of fibrin and edema were strikingly altered. The ultimate development of necrosis was diminished. The relationship

---

3. Taubenhaus, M., and Amromin, G. D., *J. Lab. and Clin. Med.,* 1950, v36, 7.

4. Dougherty, T. F., and Schneebeli, G. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 854.

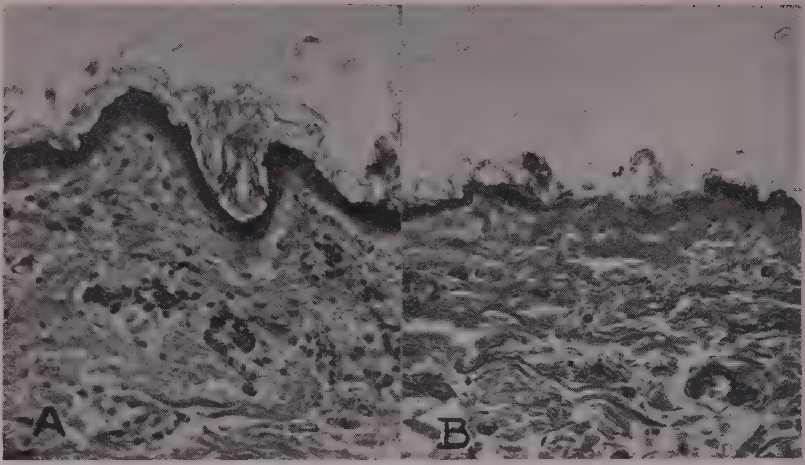


FIG. 1a.
Two hr (control): Margination and beginning migration of leukocytes.
FIG. 1b.
Two hr (cortisone): Area of necrosis in upper corium representing needle scratch. Minimal margination of leukocytes.

of these observed facts to the enhancing effect of cortisone and ACTH on certain bacterial infections is suggested.

Received March 9, 1951. P.S.E.B.M., 1951, v76.

The Plasma Potassium Level in the Newborn.* (18621)

D. P. EARLE, H. BAKWIN, AND D. HIRSCH.

From the Departments of Medicine and Pediatrics, New York University College of Medicine, New York City.

High serum potassium levels during the early weeks of infant life have been reported by several observers. Data from the literature are summarized in Table I. The values recorded are well above the normal adult level of 4.41 meq./liter, $\sigma = 0.36$ meq./liter (5). The erythrocytes contain a considerable amount of potassium which may diffuse out of the cells under certain circumstances or which may be released by hemolysis. Either of these circumstances could produce erroneously high plasma or serum potassium levels. The problem, therefore, has been reinvestigated.

Methods. Placental blood was obtained immediately after the infant was born and before the cord was severed. Blood was withdrawn through a needle inserted in the umbilical vein. Blood was obtained from young infants from the femoral vein or the superior longitudinal sinus. Dried heparin was used to prevent clotting. The blood sample was immediately centrifuged and the

---

* This work was supported in part by the Life Insurance Medical Research Fund.

1. Edelstein, F. and Yllpö, A., *Z. f. Kinderheilk.*, 1920, v27, 79.

2. Bakwin, H. and Rivkin, H., *Am. J. Obstet. and Gyn.*, 1927, v13, 68.

3. Kotikoff, J. A., *Jahrb. f. Kinderheilk*, 1933, v138, 280.

4. McCance, R. A., and Young, W. F., *J. Physiol.*, 1941, v99, 265.

5. Farber, S. J., Pellegrino, E. D., Conan, N. J., and Earle, D. P., *Am. J. Med. Sci.*, in press.

TABLE I. Serum Potassium Levels in Young Infants. Data from the literature.

Authors	No. of cases	Age	Serum potassium, meq./l Mean	Serum potassium, meq./l Range
Edelstein and Yllpö (1)	11	Placental blood	11.04	7.09-22.92
Bakwin and Rivkin (2)	20	” ”	9.63	5.75-13.00
Kotikoff (3)	14	<1 mo.	7.03	5.30- 8.39
McCance and Young (4)	12	7 to 14 days	7.65	4.25-10.08

TABLE II. Plasma Potassium Levels in Newborns and Infants.

	Plasma potassium, meq/liter Individual levels									Mean
Placental plasma	4.5	4.6	4.6	5.0	5.1	5.1	5.2	5.3	5.4	4.98
1 to 3 days old	2.7	4.8	5.1	5.1	5.3	5.8				4.89
6 to 9 days old	3.8	4.6	4.7	4.8	4.9	5.1	5.2	5.7	5.8	

plasma was separated from the erythrocytes within 20 minutes of withdrawal from the body. Specimens with more than a faint trace of hemolysis were discarded. All potassium measurements were made on an internal standard flame photometer with an error of less than 1%.

Results. The plasma potassium levels observed in 9 placental samples and in 15 infants ranging in age from 1 to 9 days are recorded in Table II. Although the mean plasma potassium levels are only slightly above the mean normal for adults, all but 2 of the 15 infants have values greater than the adult mean.

Comment. When strict attention is paid to details in obtaining plasma samples, the plasma potassium level in blood obtained from the placenta and from infants under 10 days of age is only slightly greater than the mean normal adult value. Earlier reports of potassium levels in placental serum and in infants' serum well above the normal adult average and ranging up to 23 meq./liter appear to be erroneous.

Summary. Plasma potassium levels of placental and early infantile blood samples are only slightly greater than the mean normal level for adults.

Received March 12, 1951. P.S.E.B.M., 1951, v76.

Bile Acids in Blood and Lymph in Experimental Obstructive Jaundice.* (18622)

Charles R. Reiners, Jr. (Introduced by C. G. Johnston)

From the Department of Surgery, Wayne University College of Medicine, Detroit.

While engaged in studies endeavoring to discover more of the nature of the metabolism of the bile acids in obstructive jaundice, several phenomena were observed which seem sufficiently important to report at this time. In these experiments we have employed the method of Irvin, Johnston, and Kopala(1) for determining the concentration of cholic acid in blood and lymph. This method has shown itself to be reliable, and obviates many of the difficulties encountered in the earlier methods of bile acid analysis.

I. Four dogs were employed. Under intravenous nembutal anesthesia, the common bile duct was double ligated and severed between the ligatures. The cystic duct was ligated

---

* Aided by a grant from the Parke, Davis and Co.

1. Irvin, J. L., Johnston, C. G., and Kopala, J., *J. Biol. Chem.*, 1944, v153, 439.

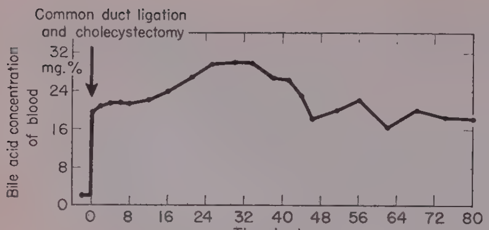


Fig. 1.

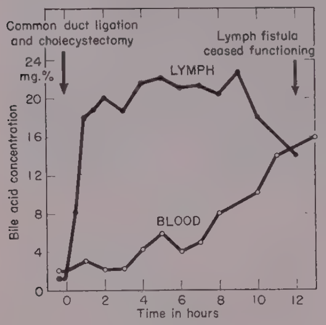


Fig. 2.

and the gall bladder was removed. One blood sample was drawn preoperatively. Postoperative samples were taken at hourly intervals for the first several hours, then at increasingly longer intervals (1 to 5 days) until the animal died. The survival periods ranged from 70 to 105 days. All dogs rapidly developed intense jaundice. They maintained their weight well for the first 30 to 60 days, after which they began to lose weight, refused to eat, and later died, apparently from inanition. They remained active and alert until the terminal period.

The bile acid concentrations in the blood of these dogs all followed a similar curve (Fig. 1). The normal concentration ranges from 1 to 3 mg %. (This is also the normal level found in humans by the use of this method.) Following complete obstruction of the biliary tree with exclusion of the gall bladder, the bile acid concentration of the blood rises suddenly from normal levels to values of 17 to 20 mg % within the first 3 to 4 hours. There is a more gradual rise over the next 20 to 30 days until a peak level of approximately 30 mg % is attained, then a very gradual decline until death supervenes. The lowest value observed was 11 mg % in the terminal phase of one animal. The other three died while the bile acids were still 18 mg % or higher.

II. Again 4 dogs were used. They were all operated upon in the fasting state. The thoracic duct was catheterized by a plastic tube where it entered the left subclavian vein. Specimens of lymph and blood were taken for baseline values. The common bile duct was then ligated and severed and the gall bladder removed as in the previous experiment. Blood and lymph samples were taken at half hour or hour intervals and analyzed for bile acids.

The results are shown in Fig. 2. The initial bile acid concentration of the lymph in these animals was the same as in normal blood (1 to 3 mg %). Following obstruction of the biliary tree and exclusion of the gall bladder the concentration became very high within one hour. The increase in the blood was much slower. It was impossible to carry this experiment farther than 8 to 12 hours because the dogs poured out such large quantities of lymph and became so dehydrated that lymph ceased to flow. The administration of intravenous saline in 2 of the dogs prolonged the flow from the functioning fistula for only a few hours.

Discussion. Other investigators(2,3) have observed rises in bile acid concentration after common duct obstruction, though not as rapidly or as large as in our experiments. In at least one of these experiments(3) the discrepancy can be partly explained by the fact that the gall bladder was left intact. Its concentrating action could account for the fact that very little of the bile acid found its way into the general circulation.

Summary. 1. Studies of the bile acid in the blood of patients with obstructive jaundice indicate that if the blood bile acid increases after obstruction it rapidly returns to normal (4). We are unable to explain this dis-

---

2. Jungner, G., Rydin, A., and Josephson, B., *Acta. Med. Scand.*, 1938, v97, 254.

3. Chabrol, E., Charonnat, R., and Blanchard, J., *Compt. Rend. Soc. de Biol.*, 1941, v135, 359.

4. Unpublished data.

crepancy between the findings in patients with obstructive jaundice and in our experimental animals. 2. The rapid increase in thoracic duct lymph following common duct obstruction confirms the work of Mayo and Greene (5) indicating that the preferential route through which bile acid reaches the blood stream is through the lymphatics.

---

5. Mayo, C., II, and Greene, C. H., *Am. J. Physiol.*, 1929, v89, 280.

Received March 12, 1951. P.S.E.B.M., 1951, v76.

Alkyl Nitrites XVI. Comparative Depressor Responses of Octyl Nitrite and Glyceryl Trinitrate. (18623)

C. Jelleff Carr, Raymond M. Burgison, Mary S. Fassell, and John C. Krantz, Jr.

From the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore

Studies from this laboratory on the pharmacology of high molecular weight alkyl nitrites (1) led to the use of octyl nitrite (2-ethyl-n-hexyl-1-nitrite) (2) in the treatment of angiospastic disease. Octyl nitrite exerts a vapor pressure of 3.3 mm at 25°. This permits the compound to be used clinically in an inhaler and administered by inhalation for the purpose of producing prompt coronary dilatation. Comparisons of the relative efficacy of octyl nitrite and glyceryl trinitrate as therapeutic agents have been made by Freedberg *et al.*(3) and Russek and Anderson(4). The latter investigators found that the inhalation from the octyl nitrite inhaler elicited a therapeutic response comparable to the sublingual administration of 1/200 to 1/300 grain glyceryl trinitrate tablet.

We have been concerned for more than a decade in the mechanism of action of the organic nitrates and nitrites(5,6). Our studies have shown that their actions on a cellular level involve different enzymic components in arterial tissue. The purpose of this study is to assess the relative potency as depressor agents of octyl nitrite and glyceryl trinitrate, and to explore their absorption.

Methods employed. Mongrel dogs weighing from 6 to 13 kg were used in these experiments. The animals were anesthetized with ether. Blood pressure was recorded from the carotid artery with a mercury manometer. Electrocardiograms were made with a "Cardiette." Intravenous injections were made into the saphenous vein; all injections were diluted to 5 cc with normal salt solution. Inhalations of octyl nitrite were achieved by removal of the tracheal ether bottle and replacing it with a 100 cc container into which 0.2 cc of octyl nitrite had been placed.

Results. In 10 dogs, octyl nitrite was administered sublingually in doses of 0.2 cc. There were 12 doses given. Not any of these elicited a depressor response, indicating that octyl nitrite is not absorbed from the buccal mucosa of the dog. Dissolving octyl nitrite in alcohol did not promote its absorption through the buccal membranes. Similarly, amyl nitrite was not absorbed sublingually. In the same dogs, glyceryl trinitrate was administered in the form of the official 1% spirit in doses of 0.2 cc. There were 11 doses given. In all of the 9 animals a depressor response was produced. There were wide variations in depressor response evoked by

---

1. Krantz, J. C., Jr., Carr, C. J., and Forman, S. E., Proc. Soc. Exp. Biol. and Med., 1939, v42, 472.

2. Krantz, J. C., Jr., Carr, C. J., and Forman, S. E., *J. Pharm. and Exp. Therap.*, 1938, v64, 302.

3. Freedberg, A. S., Spiegl, E. D., and Riseman, J. E. F., *Am. Heart J.*, 1941, v22, 519.

4. Russek, H. I., and Anderson, W. H., personal communication.

5. Krantz, J. C., Jr., Carr, C. J., Forman, S. E. and Cone N., *J. Pharm. and Exp. Therap.*, 1940, v70, 323.

6. Krantz, J. C., Jr., Carr, C. J., Bryant, H., *J. Pharm. and Exp. Therap.*, 1951 in press.

TABLE I. Depressor Responses of Octyl Nitrite and Glyceryl Trinitrate in the Dog. (Percentage fall in blood pressure).

	Octyl nitrite		Glyceryl trinitrate	
	Inhalation (.2 cc/dog)	Intravenous (1 mg/kg)*	Sublingual (.2 cc/dog)	Intravenous (.36 mg/kg)*
High	53.4	32.3	25.0	40.0
Low	16.7	9.8	8.5	25.0
Mean	33.4 $\sigma = \pm 9.4$	22.4 $\sigma = \pm 5.9$	17.0 $\sigma = \pm 5.8$	30.0 $\sigma = \pm 4.7$

* Dose of glyceryl trinitrate equivalent to that of octyl nitrite on a molar basis depending upon the nitrate and nitrite groups present in the respective molecule.

$C_3H_5(NO_3)_3 \Bumpeq 81.9\%\ 3(NO_3)$ $C_8H_{17}NO_2 \Bumpeq 28.9\%\ NO_2$

the sublingual administration of glyceryl trinitrate. The intravenous injection of the drug prior to the sublingual administration produced a refractoriness to the depressor response by buccal membrane absorption.

When octyl nitrite was placed in the inhaler bottle in quantities of 0.2 cc it evoked a marked depressor response in 10 dogs. The effect was prompt, occurring in about 10 seconds; the average fall of blood pressure was 33.4%. The duration continued until the respirator container was withdrawn. It appears therefore that depressor responses of great magnitude can be achieved with octyl nitrite over long periods of time as long as the vapors reach the alveoli. We(2) have shown previously that the coronary arteries share this response. There were no significant changes in the electrocardiograms of these dogs following the administration of these drugs with the exception of the characteristic tachycardia.

The two compounds were injected intravenously into 10 dogs at varying intervals to determine their relative depressor potencies. The assays were made on a molar basis dependent upon the nitrate or nitrite groups in each molecule (glyceryl trinitrate 81.9% nitrate; octyl nitrite 28.9% nitrite).

The data are shown in Table I.

Assays were also made on an equal weight basis to determine the minimal concentration which would elicit a depressor response (4 mm). When administered intravenously in 1 cc doses the threshold depressor concentration for octyl nitrite ranged from 1:250 to 1:1000. The threshold depressor concentration for glyceryl trinitrate when administered intravenously in 1 cc doses was found to range between 1:1000 to 1:10,000.

The data reveal that in this series of 10 dogs, glyceryl trinitrate is the more potent depressor. This obtains when the drugs are administered on an equal molar nitrite-nitrate basis. It is again manifested in the threshold concentration studies.

The studies revealed again the varying degrees of sensitivity to the depressor response which obtains in different dogs. Of special interest is the fact that sensitivity or refractoriness to a vasodilator of the nitrate type does not necessarily indicate that the same condition will obtain with the vasodilator of the nitrite type.

Summary. (1) Octyl nitrite unlike glyceryl trinitrate is not effectively absorbed through the buccal membranes of the dog. (2) Octyl nitrite is a powerful depressor agent in the dog when administered by inhalation. (3) Intravenously in the dog the depressor potencies of octyl nitrite and glyceryl trinitrate are in the ratio of 1 to 1.34 on a molar basis, depending upon the nitrite and nitrate groups respectively present in each molecule. On an equal weight basis the depressor threshold for glyceryl trinitrate was found to be between one-fourth and one-tenth that of octyl nitrite.

Received March 12, 1951. P.S.E.B.M., 1951, v76.

An Application of Autosyns to Multi-Channel Physiological Recording.*
(18624)

Hal C. Becker, Robert Hodes, Warren L. Founds, Jr., and Samuel M. Peacock, Jr.† (Introduced by Herbert P. Sarett)

From the Department of Psychiatry and Neurology, Tulane University, School of Medicine, New Orleans.

The properties of the self-synchronous motor (known by such trade names as "Autosyn", "Selsyn", etc.) have been described by Blackburn(1) and others(2,3). Bowman(4) used these devices to record mechanical myograms on a cathode ray oscilloscope. The present paper describes the application of autosyns to obtain simultaneous ink-written records of several physiological phenomena on moving paper. The recorder to be described appears to have certain advantages over the smoked-drum or ink-writing kymograph customarily employed in the physiological laboratory.

The basic elements of a typical channel of this recorder are two autosyns of the Bendix-Pioneer Type AY-1. One of these is used as a motion take-off device (synchro generator), the other is used as a driving mechanism (synchro motor) for the ink-writing pens. With the autosyns connected as shown in Fig. 1A, a rotation of the shaft of one through a given angle causes a simultaneous rotation of the shaft of the other through this same angle. Displacements ranging from less than one millimeter to more than 10 cm have been recorded by using suitable lever arms on the motion pick-up autosyn.

For slowly changing phenomena such as respiration, overall error in angle of rotation of recording autosyn shaft for a given rotation of pickup shaft is less than 2 degrees. For more rapid motion, the fidelity of the record is a function of the character of the movement. Fig. 1B shows the overall amplitude vs. frequency curve of a typical channel. This was obtained by driving the pickup autosyn with a sine wave cam connected to the shaft of a variable speed motor. The sharp resonance in the response curve at 15 cycles per second indicates that some overshoot is to be expected in the recording of rapidly decelerating motion.‡ However, in recording the knee jerk of the cat no such overshoot has been observed. Direct measurement of the

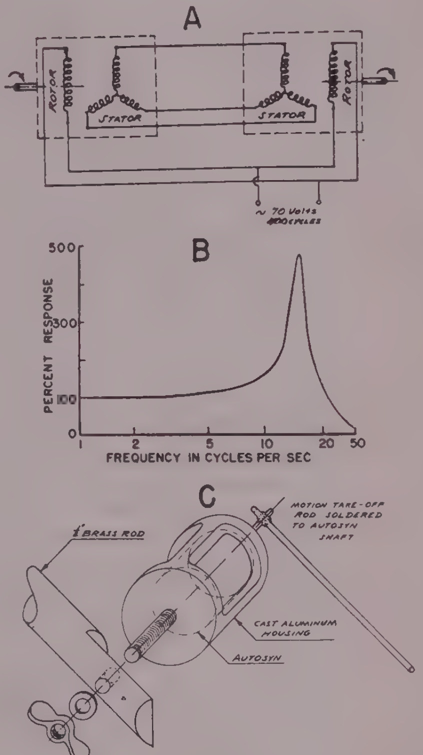


Fig. 1. A. Electrical schematic of typical channel. B. Response of typical channel as a function of frequency. C. Exploded view of autosyn mount.

---

* This work was aided by a grant from the Teagle Foundation, New York.

† Research Fellow, American College of Physicians, 1949-50.

1. Blackburn, J. F., *ed., Mass. Inst. Tech. Radiation Lab. Series,* 1949, v17, 310.

2. Greenwood, I. A.. *ed., Mass. Inst. Tech. Radiation Lab. Series,* 1948, v21, 225.

3. James, H. M., *ed., Mass. Inst. Tech. Radiation Lab. Series,* 1947, v25.

4. Bowman, R. L., *Science,* 1948, v108, 310.

excursions of the paw above and below its resting position showed that the damped oscillations in the knee jerk record of Fig. 2B represent the actual motion of the paw and are not artifacts introduced by the recording system.

Fig. 1A shows seventy volts at 400 cycles applied to the rotors of all autosyns. This voltage considerably exceeds that for which the autosyns were designed (26 volts at 400 cycles), but it is used in order to achieve sufficiently stiff coupling between motion take-off and recorder autosyns. No serious temperature rise has been observed as a result of this overload. The total 400 cycle current to a 4-channel system (*i.e.*, 8 autosyns) is 1.5 amperes at 70 volts. With power factor correction this current can be reduced to about one ampere.

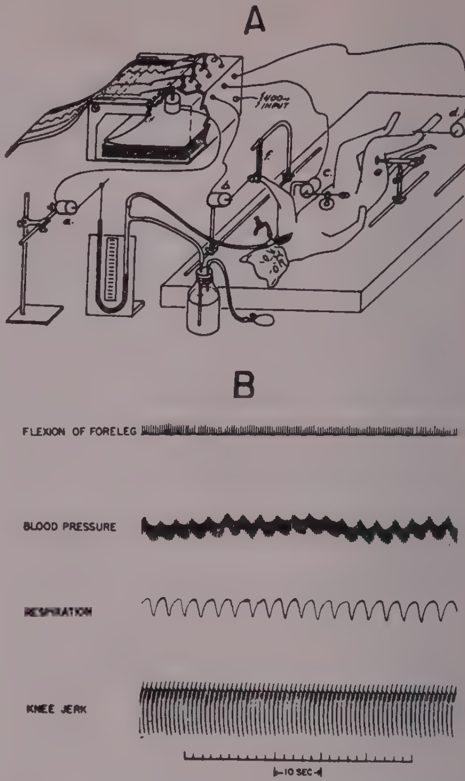


FIG. 2. A. Sketch showing autosyn take-off units, a, b, c, and d, which are used for the recording of: blood pressure from the carotid artery, movement of the foreleg evoked by stimulation of the contralateral motor cortex, respiration and patellar tendon reflex. In the figure, e shows the drill inserted into the cat's femur to stabilize the leg during prolonged elicitation of the knee jerk, and f shows support rod and elastic return for forelimb movement. B. Records from typical experiment: Foreleg flexion evoked by stimulation of the motor cortex of the opposite side 2 times per second. Blood pressure and heart rate recording from carotid artery. Note respiratoy fluctuations in pressure tracing. Pressure at highest point during inspiration. 176 mm Hg., at lowest point during expiration, 164 mm Hg. Record of knee jerk obtained by energizing a solenoid once per second, thus allowing the reflex hammer to strike the patellar tendon at that rate.

As shown in the detail of Fig. 1C, a steel rod about 1/32″ in diameter is soldered at right angles to the rotor shaft of each motion take-off autosyn and serves as the mechanical linkage to the movement to be recorded. The sensitivity of a recording channel (*i.e.*, ratio of angle of rotation of autosyn shaft to total displacement due to physiological activity) is then easily adjustable by varying the distance from the rotor shaft along the steel rod to the point of application of the displacement. This allows one to vary the amplitude of the record at will in order to facilitate initial settings or to compensate for gross physiological changes which may occur in the course of an experiment. The motion take-off autosyn is mounted on a ½″ rod which is fixed by a universal clamp to a ring stand or an upright on the animal board. The case (stator) of the pickup autosyn can be rotated with respect to the supporting rod on an axis co-axial with that of the rotor. This motion allows zero adjustment of the pen regardless of the initial angular position of the take-off shaft. In general the take-off autosyn is mounted in whatever position is necessary to cause its pickup rod to move in a plane which is perpendicular to that of the

---

‡ We have recently tested another system of recording which uses a take-off autosyn, carrier generator and phase detector feeding into a standard multi-channel electroencephalograph. In this instance the resonant peak is absent since the overall frequency response is that of the electroencephalograph itself (*i.e.*, good to about 80 cycles per second). Such a system has the added advantage of allowing the recording of mechanical displacements simultaneously with the EEG, EMG, EKG, etc.(5)

5. McCawley, E. L., Riley, J. A., and Mauro, A., *Yale J. Biol. and Med.*, 1950, v22, 411.

displacement to be recorded.

Schematic representation of a complete 4-channel recording system is shown in Fig. 2A. It utilizes 8 autosyns, 4 of which drive the recording pens, the remaining 4 of which serve as motion take-off devices. The recording autosyns are mounted beneath the top of the paper puller with their shafts extending vertically upward through holes in the top plate to connect with the pens.

A typical record showing carotid blood pressure, respiration, knee jerk, and cortically evoked forelimb movement is shown in Fig. 2B. The blood pressure record was obtained by coupling a take-off autosyn to a float riding the column of mercury in the manometer. From such a record, pulse rate and respiratory variations of blood pressure and pulse are also obtainable. Respiration was obtained by strapping a cork on the animal's chest or abdomen and placing the autosyn shaft in a slot in the cork. Knee jerk and forelimb movement were obtained by slipping the shaft of the pickup unit beneath several turns of rubber band wrapped around the extremity.

In our experience it has been possible to prepare a typical mammalian experiment more satisfactorily with the autosyn recorder than with the standard ink-writing kymograph and routine physiological recording levers, tambours, etc. We attribute this to several factors: (1) The apparatus obviates complicated mechanical, pneumatic, and hydraulic couplings between the source of the displacement and the recording system. (2) The recording system can be physically separated from the source of the movement, thus avoiding crowding around the animal table. (3) The motion pickup autosyns are easily adjusted to fit the direction of the movement. (4) The sensitivity of the autosyns makes small displacements recordable. (5) The fixed mounting of the recording pens avoids the necessity of lining up pens, or obtaining simultaneous ordinates, and facilitates the reading of the records. (6) The autosyns are simple to operate and require practically no maintenance.

Summary. An apparatus has been described which employs autosyns for the recording of displacements produced by physiological activity. The apparatus appears to have certain advantages over the kymographs customarily used for such recordings.

Received March 16, 1951. P.S.E.B.M., 1951, v76.

Effect of Adrenocorticotropin on Thyroidal Collection of I_{131} in the Adrenalectomized and Intact Rat.* (18625)

L. J. Soffer, J. L. Gabrilove, and W. R. Dorrance.†

From the Endocrine Section of the Medical Services and the Chemical Laboratory, Mount Sinai Hospital, New York City.

This report is concerned with the effect of the administration of adrenocorticotropin on the collection of I_{131} by the thyroid gland of both adrenalectomized and intact rats.

Methods. Male rats of the Wistar strain (Carworth Farms) were placed on a low-iodine minimally goitrogenic (Steenbock) diet for a period of 2 weeks(2). They were then weighed and divided into 4 groups: Group 1, control intact; Group 2, adrenocorticotropin treated intact; Group 3, control adrenalectomized; and Group 4, adrenocorticotropin treated adrenalectomized. Bilateral adrenalectomy was carried out in the usual fashion employing a dorsal approach. In addition to

---

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

† Sara Welt Fellow in Medicine.

1. Soffer, L. J., Volterra, M., Gabrilove, J. L., Pollack, A., and Jacobs, M., Proc. Soc. Exp. Biol. and Med., 1947, v64, 446.

2. Soffer, L. J., Gabrilove, J. L., and Jailer, J. W., Proc. Soc. Exp. Biol. and Med., 1949, v71, 117.

TABLE I.

Group	No. of animals	Initial body wt (g)	Final body wt (g)	Thyroid wt (mg)	Thyroidal uptake of I_{131}, %
1. Control-Intact	20	130.1 ± 2.4	146.2 ± 2.7	12.5 ± 0.4	33.2 ± 2.1
2. Intact-ACTH	19	126.9 ± 3.1	134.9 ± 4.5	12.0 ± 0.4	20.9 ± 1.8
3. Control-Adrenalectomy	11	128.4 ± 3.0	126.5 ± 3.8	10.7 ± 1.1	31.2 ± 2.3
4. Adrenalectomy-ACTH	7	129.7 ± 3.1	138.3 ± 4.7	11.3 ± 0.7	18.0 ± 0.9

$$S.E. = \sqrt{\frac{\Sigma d^2}{(n)\ (n-1)}}.$$

3 cc of 5% glucose in normal saline administered subcutaneously at the time of operation, the adrenalectomized animals were given 0.5 mg of desoxycorticosterone acetate daily by intramuscular injection. Beginning on the fifth post-operative day, 5 mg of adrenocorticotropin was administered subcutaneously daily in three divided doses for a period of 4 days to Groups 2 and 4. On the fourth day of adrenocorticotropin therapy all 4 groups of animals were given approximately 1 microcurie of I_{131} intraperitoneally. Twenty-four hours later the animals were killed rapidly en masse with ether anesthesia. The thyroid glands were dissected away carefully, weighed on a torsion balance, digested in sodium hydroxide, and analyzed for I_{131} as described previously(2).

Results. The collection of I_{131} by the thyroid gland was reduced following the administration of adrenocorticotropin in both the adrenalectomized and the intact rats. The thyroidal uptake of the radioactive isotope was the same in the control intact and adrenalectomized groups. Similarly, no difference in uptake was noted between the adrenocorticotropin treated intact and adrenalectomized animals.

Discussion. We have previously reported that the thyroidal collection of I_{131} is reduced following the injection of epinephrine and of cortisone(1,2). These studies have since been confirmed by other observers who demonstrated a decrease in the uptake of I_{131} by the thyroid following other forms of stress and following the injection of adrenocorticotropin as well as of cortisone to the intact animal (3-5). More recently we have demonstrated (6) that the thyroid hyperplasia induced by the feeding of propylthiouracil can be inhibited by the administration of adrenocorticotropin. Under the conditions of this latter experiment, cortisone, however, induced no demonstrable change.

The experimental studies now reported demonstrate that adrenocorticotropin reduces the collection of I_{131} by the thyroid gland even in the absence of the adrenal cortex. This would suggest that this is mediated by means of a direct inhibitory effect of adrenocorticotropin on the adenohypophyseal secretion of thyrotropin. It is unlikely that the action of adrenocorticotropin is on the thyroid gland, since recently reported experiments indicate that adrenocorticotropin does not prevent the action of thyrotropin on the thyroid gland(7).

These observations may explain the seemingly paradoxical results reported in patients with Addison's disease treated with cortisone (7). In these subjects there is frequently observed a temporary increase in the thyroidal I_{131} accumulation gradient followed by a decrease to values below that noted in the untreated state. It is possible that the ad-

---

3. Williams, R. H., Jaffe, H., and Kemp, C., *Am. J. Physiol.*, 1949, v159, 291.

4. Paschkis, K., Cantarow, A., Eberhard, T., and Boyle, D., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 116.

5. Money, W. L., Kirschner, L., Kraintz, L., Merrill, P., and Rawson, R. W., *J. Clin. Endocrinol.*, 1950, v10, 1282.

6. Gabrilove, J. L., and Soffer, L. J., *J. Clin. Invest.*, 1950, v29, 814. (abstract).

7. Hill, S. R., Reiss, R. S., Forsham, P. H., and Thorn, G. W., *J. Clin. Endocrinol.*, 1950, v10, 1375.

ministration of cortisone may first result in a decrease of adrenocorticotropin secretion and a consequent removal of the inhibiting effect of this hypophyseal fraction on thyrotropin secretion. Subsequently the direct inhibiting effect of cortisone on thyrotropin secretion comes into play.

The results of the studies reported from this laboratory would suggest that the phenomenon of stress is characterized by a discharge of adrenocorticotropin and thyrotropin from the adenohypophysis, and that the subsequent secretion of thyrotropin is inhibited both by adrenocorticotropin and by certain fractions of the adrenal cortex.

Summary. The administration of adrenocorticotropin results in a decreased collection of I_{131} by the thyroid gland of both the intact and adrenalectomized rat, suggesting that adrenocorticotropin inhibits the adenohypophyseal secretion of thyrotropin.

Received March 16, 1951. P.S.E.B.M., 1951, v76.

Pathological Changes in Rabbits from Repeated Intravenous Injections of Periston (Polyvinyl Pyrrolidone) or Dextran. (18626)

ARTHUR A. NELSON AND LEHMAN M. LUSKY.

From the Division of Pharmacology, Food & Drug Administration, Federal Security Agency, Washington, D.C.

The paucity of information in the American literature on the histopathological changes resulting from intravenous injections of periston (polyvinyl pyrrolidone), a blood plasma substitute, prompts the presentation of this report. In addition to clinical experience with periston, the histological changes in animals and in a few infants have been reported(1). Dextran, another plasma substitute, has been stated to cause no morphological changes in animals injected repeatedly(2).

We have administered to rabbits, over a 2 month period, 16 intravenous injections of 10 cc/kg of 6% dextran* in distilled water, or 3½% periston† in distilled water, or 10% dextrose in physiological saline solution (Table I). The ear veins were used, and each injection took about 10 minutes. No systemic effects, even of a minor nature, were observed during the experimental period. One male rabbit on dextrose died from dysentery 2 weeks after the start of the experiment; this death could not be attributed to dextrose. No blood counts or blood chemical studies were done on these animals.

Gross pathology. In the animals given dextran there were no gross abnormalities that could be attributed to that agent. In the group given periston, the spleen showed definite slight enlargement (Table I). Except for the increase in size, there was no difference in the appearance of the spleen. The periston animals had an average of 0.70 g of spleen per kg animal, while the values for dextran and dextrose were 0.40 and 0.41 respectively. In the dextrose group, there was possibly slight renal enlargement (Table I). With respect to "spontaneous" lesions and parasitism, the rabbits on the whole were very free from these conditions.

Microscopic pathology. The one outstanding lesion of the entire experiment was a foam cell storage phenomenon in the periston animals; this was seen best in the spleen, and in lesser degree but sometimes prominently in several other structures. In the *spleen* the foam cells made up an estimated ⅓ to ½ of the volume of the organ. The foam cells had reticular type nuclei, basophilic and markedly vacuolated cytoplasm, and were from 15 to 100 (usually 20-25) μ in diameter, the larger ones generally being multinucleated. The foam cells and especially their vacuoles contained periston or some near derivative, the

---

1. Schoen, H., *Klin. Woch.*, 1949, v27, 463.
2. Thorsén, G., *Lancet*, 1949, v256, 132.

* Pharmacia "Macrodex", lot 153.

† General Aniline and Film Corp., lot 1687-202.

TABLE I. Weights of Rabbits and of Various Organs.

Rabbit No.	Sex	Substance injected	Wt of rabbit, kg		Wt in g		
			Start	Finish	Liver	Kidneys	Spleen
1	M	6% Dextran	2.52	3.32	101	16.0	1.27
2	M		2.50	3.50	114	14.3	1.60
3	M		2.92	3.22	130	19.1	1.32
4	M		2.63	3.08	114	14.5	0.90
5	F		2.52	3.12	100	16.0	1.48
6	F		2.75	3.50	101	16.5	1.43
		Avg	2.64	3.29	110	16.1	1.33
7	M	3½% Periston	2.38	2.88	96	16.5	2.70
8	M		2.80	3.32	95	18.5	2.45
9	M		2.58	3.40	125	21.0	1.50
10	M		2.36	3.10	110	16.0	1.60
11	F		2.63	3.08	111	15.5	2.55
12	F		2.26	3.02	97	20.0	2.47
		Avg	2.50	3.13	106	17.9	2.21
14	M	10% Dextrose	3.25	3.72	128	23.0	1.05
15	M		2.83	3.02	73	16.3	0.97
16	F		2.83	3.47	129	24.3	1.35
17	M		3.44	3.60	118	23.5	1.80
18	F		2.62	3.15	93	16.5	1.75
		Avg	2.99	3.39	108	20.7	1.38

identification of which will be discussed further on. Some of the larger foam cells also contained a few leucocyte fragments. Among other organs than the spleen, the foam cell storage phenomenon in the periston animals was seen in moderate degree in the *lymph nodes, bone marrow,* and *adrenal medulla,* and in lesser degree in the *liver, lungs,* and *thymus.* These structures were otherwise unaffected in all groups of animals. It will be observed that the distribution of the foam cells is essentially that of the reticulo-endothelial system, or that involved in intravital staining. *Periston,* apart from the foam cell storage phenomenon, caused slight testicular atrophy, slight epithelial swelling and rarefaction in the distal portions of the proximal convoluted renal tubules and in the choroid plexus of the brain, and (by inspection) a slight "shift to the left" of leucocytes in the bone marrow, with a possible slight reduction in the myeloid/erythroid ratio. *Dextran* caused very slight testicular atrophy, the same leucocyte shift, and a slight reduction in the M/E ratio. *Dextrose* caused no changes. In *all groups,* heart, gall bladder, pancreas, small intestine, thyroid, prostate, seminal vesicle, uterus, ovary, urinary bladder, and voluntary muscle showed no effect of treatment.

Special stains. Periston is highly soluble in water and alcohol, so that paraffin or even frozen sections contained little of the stored material in the foam cells. The stored material was well demonstrated, however, in the foam cells in unfixed imprints. About 2 minutes after a few drops of Gram's iodine solution and a coverglass were applied, the foam cells showed numerous deep brown small and large globular masses, while other structures (bone marrow) were relatively unstained. Glycogen as the material taking up iodine was ruled out by the total lack of staining of any material within the vacuoles in alcohol-fixed tissue to which a periodic acid-Schiff stain was applied, and with which liver glycogen was deeply stained. Other negative tests on the foam cells in addition to that for glycogen were those for doubly refractive material, for fat, and for metachromasia, using appropriately fixed material and standard technics. In frozen sections of formalin fixed material, the foam cells (at least if fixation had been fairly recent) still contained a fair amount of iodophilic material, but paraffin sections of similar material gave almost no reaction, as was also true with alcohol fixed material. However, in paraffin sections of Zenker fixed material the foam cells took iodine somewhat selectively.

Discussion. Our results are presented because of current interest in these substances, and because little if any report of animal studies has appeared in the American literature. A report of results with an older type of dextran has appeared(3). The Swedish dextran has been stated to cause no lesions in animals(2), and we found very little effect. The lesions observed by us following periston injections are of the type and order of magnitude already reported. The foam cell accumulations caused by periston are said to disappear after several weeks(4). Periston in our material did not cause the arterial alterations, in addition to foam cell storage, noted with some previously tried plasma substitutes(5).

Summary. Seventeen rabbits were each given 16 intravenous injections of 10 cc/kg of one or another plasma substitute over a 2-month period. The solutions used were 6% dextran in distilled water, 3½% periston (polyvinylpyrrolidone) in distilled water, and 10% dextrose in physiological saline solution. No effects of treatment on behavior or body weight were noted. At autopsy, there was slight enlargement of the spleen, on the average about 70%, in the periston animals. Kidney and liver weights were not affected except for a possible slight increase in kidney weight with dextrose. On detailed microscopic examination, the one outstanding lesion was a foam cell storage of periston or some near derivative. Certain other and minor lesions were caused by periston and dextran, and none by dextrose.

---

3. Goldenberg, M., Crane, R. D., and Popper, H., *Am. J. Clin. Path.*, 1947, v17, 939.

4. Barfuss, F., and Eichler, O., *Arch. f. exp. Path. u. Pharm.*, 1949, v206, 346.

5. Hueper, W., *Arch. Path.*, 1942, v33, 267.

Received March 19, 1951. P.S.E.B.M., 1951, v76.

Chronic Toxicity of a Purified Extract of *Veratrum viride*.* (18627)

JAMES GOURZIS AND ROBERT O. BAUER. (Introduced by George L. Maison)
From the Department of Pharmacology, School of Medicine, Boston University.

Examination of available literature failed to reveal long term chronic toxicity studies on *Veratrum viride* or its extracts. Perdue *et al.* (1) reported a short term (8 week) paired feeding study using powdered root of *Veratrum viride* in Long-Evans rats. They concluded severe toxicity was not encountered until about 3% was included in the diet, and that the toxicity could be related to inanition.

Since clinical use of veratrum preparations in the treatment of hypertension requires the ingestion of the drug over a period of years it seemed desirable to observe the effects of feeding this drug over 12 months to rats and dogs. The use of the purified extract of *Veratrum viride* (Veriloid†) for the present study offered the first opportunity to feed mixtures of hypotensive potency known to be uniform(2).

Method. Rats: Albino rats (Wistar Strain), 30 days of age, weighing 64.6 ± 12.9 g were divided into 5 groups of 30 (15 females and 15 males) animals. The rats were fed a modified Steenbock's diet(3) and water *ad libitum*. The drug was incorporated in the diet of the first 4 groups while the fifth group served as the control. In 3 of the experimental groups (low, medium and high dose) the drug concentration was gradually increased over a 4- to 10-week period until 7.3, 11 and 19 mg % had been attained in the respective diets. The fourth group (intensive group) were, from the beginning, given diet

---

* Grateful acknowledgement is made to Riker Laboratories for financial support of this study.

1. Perdue, Adaline S., Bell, Francis, Stearnes, W. R., Hitchcock, Philip and Teague, R. S., *J. Am. Pharm. A.* (Scient. Ed.) 1950, v39, 91.

† Trademark of Riker Laboratories, Los Angeles, Calif. A purified reproducible hypotensive extract of *Veratrum viride* assayed in the dog.

2. Maison, G. L. and Stutzman, J. W., *Arch. internat. de pharmacodyn. et de therap.*, 1951, v85, 357.

3. Steenbock, H., *Science*, 1923, v58, 449.

such that the daily dose of drug would be in the region of the acute oral LD50 for the rat (12.25 (10.6-14.2) mg/kg)(4). During the first 6 months this latter group ate a diet containing 11 mg % of drug and during the last 6 months the diet contained 19 mg %. Body weight, food and drug consumption, appearance and behavior of the rats were recorded weekly. Complete blood counts and hemoglobin determinations were recorded for ⅓ of the animals in each group before the experiment, at six months and at termination. Dogs: Mongrel dogs (8 female and 7 male), weighing 9.8 ± 2.4 kg (between 6.8 and 15.4 kg), separately caged, were given 15 g/kg ground horse meat three times daily and allowed water *ad libitum*. After a 28-day control feeding period the standard Veriloid powder was incorporated into the diet of 12 animals while 3 continued as controls. The diet was prepared fresh for each meal, the drug being mechanically dispersed through the meat. The quantity of Veriloid added to each meal was determined on the basis of the individual animal's recent history of emesis and diet refusal. Daily recording of food and drug consumption, incidence of emesis and the general behavior of the dog were made. The animals were weighed weekly. Complete blood counts, hemoglobin determinations, routine urinalysis and serum bilirubin values were obtained during the control period, at six and twelve months. A female and two males were maintained as dietary, weight and pathological controls.

Pathologic examination. At 6 months, 25 rats (5 animals from each group) and 5 dogs (1 female control, 2 female and 2 male Veriloid fed dogs) and at twelve months 36 surviving rats and 7 dogs (1 male control, 1 male and 5 female Veriloid fed dogs) were sacrificed‡ for macroscopic and microscopic examination. Weights of the body, brain, lungs, heart, liver, spleen, kidneys and testes were recorded. Histologic sections of the following organs were made at random: brain, lung, heart, liver, pancreas, adrenals, spleen, gastro-intestinal tract, kidneys, testes, ovaries, sternum, muscle, skin and thymus.

Results. Rats: A progressive failure to gain weight (depressed growth) resulted in the 4 experimental groups ingesting graded doses of Veriloid (Fig. 1). Food and drug consumed and mortality in the 4 experimental groups as well as the control groups are recorded in Table I. In the experimental groups, almost three-quarters of the mortality occurred in the first 12 weeks during which time the animals were in their active phase of growth. Animals dying in the drugged and control series were indistinguishable. These animals stopped eating, lost weight, lost the pink ear color; their coats became shaggy and unkempt. Death followed in a few days.

Dogs. The animals fed the drug lost weight during the first 6 months of the experiment, (from 10.0 ± 2.0 kg to 8.0 ± 2.2 kg). During the second 6 months greater care was taken to reduce emesis and food rejection by individual dosage control. At the conclusion of 12 months the 5 surviving dogs had recovered and exceeded their starting weight. The 3 control dogs consistently

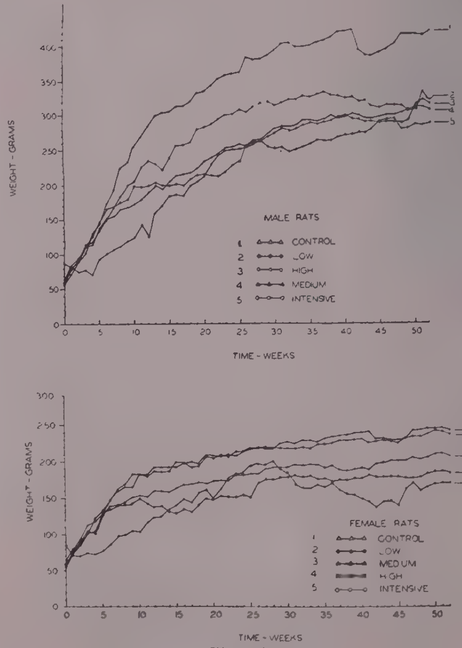


FIG. 1.
Growth curves of rats fed Veriloid.

---

4. Bauer, R. O., *Fed. Proc.*, 1950, v9, 1.

‡ Dogs sacrificed by exsanguination during pentobarbital anesthesia, rats by guillotine.

TABLE I. Summary of Weight, Mortality, Food and Drug Intake of Rat Groups Fed Veriloid for 12 Months.

		Start		1st 6 mo.			2nd 6 mo.					
				No. animals			No. animals					
Group	Sex	No. animals	Mean wt S.D., g	Dead*	Surviving	Mean wt with S.D. (g)	Dead*	Surviving	Mean wt with S.D., (g)	Drug in diet, mg %	Mean food intake and S.D., g/rat/day	Mean drug intake and S.D., mg/kg/day
Control	M	13	55 ± 6	4	9	364 ± 25	0	7	424 ± 17	—	15.2 ± 2.4	—
Low	M	16	63 ± 9	3	13	308 ± 22	1	9	323 ± 74	7.3	13.5 ± 1.7	3.2 ± 0.2
Medium	M	16	61 ± 6	2	14	258 ± 51	0	12	311 ± 52	11	12.5 ± 1.6	5.0 ± 1.2
High	M	16	59 ± 4	3	13	233 ± 44	1	10	319 ± 32	19	11.9 ± 1.6	8.0 ± 2.4
Intensive	M	15	87 ± 2	13	2	252 ± 14	0	2	291 ± 11	11–19†	14.9 ± 4.2	10.2 ± 2.1
Control	F	16	56 ± 9	1	15	219 ± 14	1	11	249 ± 24	—	12.0 ± 1.3	—
Low	F	13	65 ± 11	1	12	218 ± 23	0	10	244 ± 28	7.3	10.6 ± 0.9	3.2 ± 0.2
Medium	F	14	60 ± 5	1	13	187 ± 27	1	9	211 ± 19	11	10.7 ± 1.0	5.9 ± 1.6
High	F	14	60 ± 4	7	7	151 ± 28	0	4	189 ± 62	19	9.8 ± 1.7	9.9 ± 3.2
Intensive	F	17	83 ± 1	11	6	196 ± 8	4	2	175	11–19†	10.8 ± 2.9	10.5 ± 3.0

* Does not include rats sacrificed for pathologic section.
† Explanation in text.

gained weight during the period of observation (2 for 6 months, 1 for 12 months). In general the daily food consumption was smaller and more variable for the drugged than for the control dogs (Table II).

Pathologic findings. Macroscopic or histologic changes attributable to Veriloid administration were not observed. Liver changes were seen in both control and experimental rats and dogs. These consisted of peri-portal round cell infiltration and portal congestion. Thickening of pulmonary alveolar walls by round cell infiltration was observed in sections from control as well as drugged rats and dogs. These changes were regarded as non-specific inflammation. All other organs observed displayed normal histologic structure. Organ weights of the experimental animals did not differ significantly from those of the controls. There were no hematologic or urinary abnormalities at six or twelve months.

Discussion. The graded depression of the weight-growth curves of the rats resulting from graded doses of Veriloid suggested the presence of chronic toxic properties in the drug. The failure to show progressive pathologic changes with increasing doses implies that these toxic properties are non-specific.

These findings are in accord with the hypothesis of deficient nutrition advanced by Perdue *et al.*(1). The lower food consumption of the drugged animals is concordant. In the dogs the failure to eat was correlated with occurrence of emesis and in the dogs which died severe inanition preceded death.§

The mean daily drug intake of the several groups of rats (low, medium, high and intensive) approximated 30, 50, 80 and 100 times the average daily dose/kg for patients as reported by Wilkins *et al.*(5). These data then suggest a respectable margin of safety for the agent. Excessive quantities of the drug apparently would be required to produce this non-specific toxicity in man and the produc-

---

§ This statement describes all cases except No. 4A which died after only 3 weeks exposure to drug. Post mortem was not obtained because of autolysis.

5. Wilkins, R. W., Stanton, J. R., Freis, E. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 302.

TABLE II. Summary of Weight, Mortality, Food and Drug Consumption in Dogs Fed Veriloid.

	Wt, kg				Duration drug exposure, days	Mean food intake, g/kg/day	Mean drug intake, μg/kg/day	
Dog	Control	6 mo.	12 mo.	At death				Remarks
Control	15.2*	17.2*	—	15.8*	(273)	36.3 ± 4.9	Control	Death from acute gastric perforation and hemorrhage
,,	10.4*	10.4*	—		(200)	42.8 ± 5.8	,,	
,,	6.8*	8.7*	9.5*		(378)	40.8 ± 6.2	,,	
4a	7.3†	—	—	7.7	21	36.0 ± 7.8	110 ± 23	Delivered pups after 30 days drug exposure
4b	8.3†	—	—	5.3	45	16.1 ± 17.0	59 ± 50	
5	8.6	6.4	8.6	—	350	51.4 ± 26.4	203 ± 68	
6	8.0†	—	—	5.2	138	36.7 ± 6.8	220 ± 53	
7	12.0†	10.9	—	6.0	350	37.6 ± 8.9	195 ± 59	
8	10.9†	6.8	—	6.1	190	33.5 ± 12.3	173 ± 36	
9	7.9	6.4	8.4	—	350	39.9 ± 8.9	223 ± 62	
10	6.8	6.0	8.4	—	350	41.4 ± 10.9	227 ± 57	
11	8.8	7.9	10.9	—	350	40.0 ± 13.2	158 ± 45	
12	11.4	7.8	—	—	172	28.0 ± 12.7	114 ± 39	
13	12.1	7.8	11.6	—	350	33.6 ± 8.6	143 ± 56	
14	12.0	12.3	—	—	172	34.9 ± 6.8	198 ± 57	
Mean	9.8	8.0	9.6	—				
S.D.	2.4	2.2	1.6	—				
Mean†	9.3	—	—	6.1				
S.D.†	2.0	—	—	1.0				

* Not included in mean or S.D.

† Animals which died in course of experiment.

tion of emesis would prevent such occurrence.

Summary. (1) A purified extract of *Veratrum viride,* Veriloid, was fed to growing rats and adult dogs for 12 months. (2) A graded retardation of growth was observed in the rats consuming 3, 5, 8 and 10 mg/kg per day of the drug. No such gradation of pathologic change was found. (3) Weight loss and emesis resulted in the dogs. The mean daily intake for this species was limited to 0.19 mg/kg by emesis and failure of animals to eat. (4) No specific organ toxicity was found in either species.

Received March 20, 1951. P.S.E.B.M., 1951, v76.

Effect of ACTH on Decreased Serum Inorganic Phosphorus Induced by Insulin and Glucose. (18628)

Francisco De Venanzi.*

From the Departmento de Patologia General y Fisiopatologia. Instituto de Medicina Experimental, Universidad Central de Venezuela, Caracas.

The mechanism of the diabetogenic action of ACTH (adrenocorticotropic hormone) and of 11 oxi-cortico-steroids is little known. Ingle and Thorn(1) have been able to secure evidence against the possibility of a simple increase in neoglucogenesis. Ingle(2), demonstrated the decrease in peripheral utilization of glucose determined by adreno-cortical extracts in the eviscerated rat treated with insulin and his experiments were in harmony with the inverse phenomenon of the facilitation of utilization induced by adrenalectomy in the eviscerated rat as described previously by Russell(3). The nature of these changes is not clear at the present time. Conn *et al.* (4) in diabetes produced in man by the administration of ACTH, have afforded proof of the participation of a decrease of SH groups as reflected in a marked drop in blood glutathione levels. At the same time, an increase in uric acid excretion in the urine was noticed. Stetten(5) suggested that the inhibition of hexokinase may be the responsible factor, but this phenomenon takes place in the muscle extracts of diabetic animals and not in the ones from normal animals(6).

---

* With the technical assistance of Dra. Marianna de Masin. We wish to express our gratitude to the Eli Lilly Co. for the generous donation of the insulin-zinc crystals used in this experiment.

1. Ingle, D. J., and Thorn, G. W., *Am. J. Physiol.,* 1941, v132, 670.

2. Ingle, D. J., *Ann. New York Acad. Sci.,* 1949, v50, 576.

The administration of dextrose in the normal organism is accompanied by a decrease in serum inorganic phosphorus(8,9). This decrease does not occur, and if so, very slightly in the diabetic subject(9,10). The administration of insulin in the normal or pancreatectomized animal is followed by the decrease(7,8,11-13). Most of the experimental work tends to show that the phosphorus leaving the circulatory stream is used for phosphorylization purposes(14,15). Soskin and Levine(16) attributed the decrease in inorganic phosphorus to esterification processes taking place within the blood. Arterio-venous differences in serum inorganic phosphorus are greater after the administration of dextrose in the dog(17). It may be that the decrease in inorganic phosphorus could be used as an approximate index of phosphorylization processes. The treatment with ACTH produces in the dog an inhibition of the mentioned phenomenon after the venous injection of glucose solution(18) that is not parallel with the disturbance of the carbohydrate tolerance; with certain dosages it tends to appear and disappear later than the diabetic type of blood sugar curve(18). It would be interesting to discover if this perturbation of the phosphorus decrease is due to an insulin deficiency or is also a reflection of a peripheral disturbance. The administration of insulin associated with the same amount of glucose used in the mentioned experiments(18) in order to avoid the secondary epinephrine liberation, may give some indications in this regard. The results of such work are here reported.

Methods. Six normal and well nourished dogs were used. They were trained by previous repeated veni-punctures in order to avoid fear and anger reactions. The test performed during the different stages of the experiment consisted of taking a venous blood sample under fasting conditions and giving immediately after an injection by vein of 1 ml per kg of body weight of a 50% glucose solution containing 1 U of insulin zinc crystals per every six ml. Thirty, forty-five and sixty minutes after the injection, venous blood samples were obtained. All the samples were analyzed in duplicate for serum inorganic phosphorus with the procedure of Fiske and Subbarow(19), slightly modified and for blood sugar with the method of Nelson(20). All the absorption readings were done in a Coleman Model 6 Spectrophotometer.

The test was performed in the six dogs and then they received the ACTH. Three dogs were given 20 mg daily in one injection for

---

3. Russell, J. A., *Am. J. Physiol.*, 1943, v140, 98.
4. Conn, J. W., Louis, L. H., and Wheeler, C. E., *J. Lab. Clin. Med.*, 1948, v33, 651.
5. Stetten, D., *J.A.M.A.*, 1946, v132, 373.
6. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, v168, 583.
7. Perlzweig, W. A., Latham, E. and Keefer, C. S., Proc. Soc. Exp. Biol. and Med., 1923-24, v21, 33.
8. Harrop, G. A., and Benedict, E. M., *J. Biol. Chem.*, 1924, v59, 683.
9. Bolliger, A. and Hartman, F. W., *J. Biol. Chem.*, 1925, v64, 91.
10. Hartman, F. W., and Bolliger, A., *J. Am. Med. Assn.* 1925, v85, 653.
11. Wigglesworth, V. B., Woodrow, C. E., Smith, W., Winter, L. B., *J. Physiol.*, 1923-24, v58, 327.
12. Winter, L. B., and Smith, W., *J. Physiol.*, 1923-24, v58, 327.
13. Blatherwick, N. R., Bell, M., and Hill, E., *J. Biol. Chem.*, 1924, v56, 241.
14. Sacks, J., *Science*, 1943, v98, 388.
15. Kaplan, N. O. and Greenberg, D. M., *J. Biol. Chem.*, 1943, v150, 479; *Ibid.*, 1944, v156, 511.
16. Soskin, S. and Levine, R., *Carbohydrate Metabolism*, University of Chicago Press, Chicago, 1946.
17. De Venanzi, F. and Podrizki, A., *Acta Cientif. Venez.*, 1950, v1, 65.
18. De Venanzi, F., *Acta Cientif. Venez.*, in press.
19. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.
20. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

TABLE I. Means and Standard Errors of the Changes of Blood Sugar and Serum Inorganic Phosphorus Taking Place After the Venous Administration of Dextrose (0.5 g/kg body wt), and Insulin (1 U/3 g dextrose) in Dogs Treated with ACTH.

Before ACTH	At end of ACTH	8 days after	28 days after
△ 30′	△ 30′	△ 30′	△ 30′
G. —19 ± 5.1	G. —10 ± 6.8	G. —5† ± 5.6	G. —25 ± 6.8
P. —1.16 ± 0.12	P. —1.10 ± 0.24	P. —0.84* ± 0.09	P. —0.76* ± 0.13
△ 45′	△ 45′	△ 45′	△ 45′
G. —15 ± 3.2	G. —19 ± 6.2	G. 4* ±8.6	G. —12 ± 4.1
P. —1.53 ± 0.17	P. —1.22 ± 0.31	P. —0.71* ± 0.05	P. —0.75* ± 0.17
△ 60′	△ 60′	△ 60′	△ 60′
G. —14 ± 2.9	G. —7† ± 3.08	G. 0.16* ± 5.2	G. — 6* ± 2.9
P. —1.48 ± 0.13	P. —1.04 ± 0.36	P. —0.80* ± 0.07	P. —0.88* ± 0.15

* Statistically significant.
† Moderately significant.

The averages are expressed in terms of differences in mg/100 ml in relation to the initial values.

9 days and the other three were injected twice a day with 20 mg for 5 days (ACTH Armour). The last day of the treatment the test was carried out and then 8 days and 28 days after. All figures were expressed as differences from the fasting value of blood sugar or serum inorganic phosphorus. The statistical significance of the changes found in the different periods in relation with the test done before the treatment with ACTH was studied using the "t" test of Fisher(21).

Results. Initial values for blood sugar did not change significantly and the serum inorganic phosphorus showed a significant drop 8 and 28 days after stopping the treatment (22).

In Table I and Fig. 1 the results are presented.

Discussion. As shown in relation to the effect of the ACTH treatment on the decrease in serum inorganic phosphorus induced by the administration of glucose(18), it was found in the experiments that the injection of insulin plus glucose does not determine a normal decrease. This fact shows that the lack of the usual response of inorganic phosphorus to glucose administration under ACTH treatment does not mean that insulin is not sufficiently produced. The disturbance is probably mainly peripheral. If according to some evidences previously mentioned the inorganic phosphorus disappearing from the serum is used in the phosphorylization processes, the found results may be an indication of the *in vivo* inhibition of such processes determined by stimulation of the adrenal cortex.

It is interesting to point out the lack of parallelism between the changes in blood sugar and serum inorganic phosphorus after the injection of insulin and dextrose induced by the ACTH treatment, because it gives an indication about their relative independence.

A latent period is required after the beginning of the ACTH administration in order to produce the defect of the fall in serum inorganic phosphorus. The inhibition was greater 8 days after the end of the treatment and still noticeable 28 days after when the blood sugar values were already practically normal.

Summary. The effect of ACTH on the decrease of serum inorganic phosphorus induced by the administration of glucose and insulin in the normal dog has been studied. Three dogs were given 20 mg of ACTH daily in one injection for 9 days and the other 3 were injected twice a day with 20 mg for 5 days. A significant reduction of the decrease of serum inorganic phosphorus produced by the insulin and dextrose administration was registered 8 days after the end of the treatment and persisted, but not as marked, 28 days after. The defect in the hypoglycemic action of insulin was noticed at the end of the treatment, was more marked 8 days after and almost disappeared 28 days later. The results show that the inhibition of the decrease in serum inorganic phosphorus after the administra-

---

21. Fisher, R. A., *Statistical Methods for Research Workers,* Oliver and Boyd, Edinburgh 1941.
22. Masin, M., *Acta Cientif. Venez.* in press.

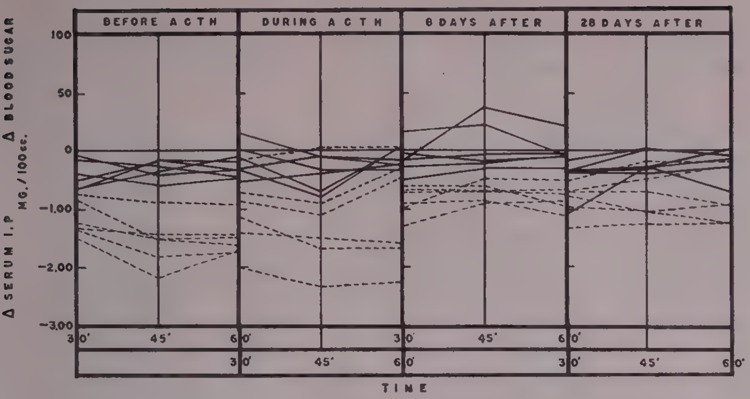


Fig. 1.

The individual variations of blood sugar (solid lines) and serum inorganic phosphorus (dotted lines) from the initial values during the different periods of the experiment.

tion of glucose alone(18) that occurs under the influence of ACTH is not due to a deficiency in insulin production. It was possible to observe that the changes of blood sugar tended to appear and disappear sooner than the modifications in inorganic phosphorus which suggest that they are independent in its mechanism although closely related phenomena. It may be that these experiments could be interpreted as an *in vivo* reduction of the rate of phosphorylization induced by adreno-cortical stimulation.

Received March 20, 1951. P.S.E.B.M., 1951, v76.

Effect of Renal Venous Occlusion on Intrarenal Pressure.* (18629)

H. G. Swann, A. V. Montgomery, and James S. Lowry.

From the Carter Physiology Laboratory, University of Texas Medical Branch, Galveston.

When the veins leaving a tissue are occluded, the hydrostatic pressure from the heart is in part transmitted into the tissue. Burch and Sodeman(1) and Wells, *et al.*(2) found an increase in subcutaneous tissue pressure of 1-3 cm H_2O during prolonged venous congestion. In muscles the same occurs: Wells, *et al.*(2) report that in venous congestion, the intramuscular pressure of the gastrocnemius rises 20 cm H_2O and that of the soleus rises 50 cm H_2O. In the case of the kidney, we have reported that renal venous occlusion causes a pronounced rise in the pressure within the kidney parenchyma(3,4). Gottschalk(5) has also studied the same phenomenon, reporting that the intrarenal pressure does not rise with partial renal venous occlusion until the renal venous pressure exceeds the preexisting interstitial pressure. The present study will describe further experiments in which the effect of both partial and total renal venous occlusion on intrarenal

---

* Supported in part by a research grant from the M. D. Anderson Foundation, Houston.

1. Burch, G. E. and Sodeman, W. A., *J. Clin. Invest.*, 1937, v16, 845.

2. Wells, H. S., Youmans, J. B., and Miller, D. G., *J. Clin. Invest.*, 1938, v17, 489.

3. Bush, W. L., Coffman, G. M., Montgomery, A. V., and Swann, H. G., *Texas Reports on Biol. and Med.*, 1949, v7, 492.

4. Montgomery, A. V., *Fed. Proc.*, 1950, v9, 91.

5. Gottschalk, C. W., *Am. J. Physiol.*, 1950, v163, 716.

pressure was measured.

Methods. Dogs under sodium pentobarbital anesthesia were used. After exposing the kidney, the intrarenal pressure was measured by the transduced equilibrium method of Swann, Montgomery, Davis and Mickle(6); this entails injecting a small volume of fluid into the kidney by means of a nearly isometric manometer. When the manometer ceases moving, it has stopped forcing fluid into the tissue; its pressure at this point, which is optically recorded, is a measure of the intrarenal pressure; the latter will be designated, for purposes of brevity, as the IRP. Arterial blood pressure was measured, after cannulation of the carotid artery, by optical methods employing a glass Bourdon tube manometer (7). The renal vein was partially or totally occluded by a tourniquet, the degree of occlusion being controlled by the number of turns made on the tourniquet. The pressure in the renal vein on the kidney side of the occlusion was measured thus: a stiff plastic ("Saran") tube, with a 2 mm bore, filled with heparinized saline, was inserted into the right external jugular vein and then manipulated down the vena cava and into the left renal vein. Its tip was set on the kidney side of the occluding tourniquet. The pressures in this tube were also recorded by a Bourdon tube manometer. They will be designated by the abbreviation VP_R.

Results. Total occlusion. When the renal vein was totally occluded, the IRP was instantaneously elevated, as shown in Table I, from a mean of 25 mm Hg up to 76 mm Hg. In 5 out of the 11 dogs (Nos. 2, 8, 9, 10 and 11), the IRP rose to approximately the simultaneous diastolic pressure, but in the remaining 6, the IRP increased to a level definitely less than the diastolic pressure. In total venous occlusion the rise in IRP cannot in fact be closely related to any component of the simultaneous arterial blood pressure: neither to the systolic nor to the diastolic nor to the pulse nor the average nor to the product of pulse rate times pulse pressure, as inspection of the table will reveal. But it does rise to the same pressure, as shown by the encircled points in Fig. 1, as the simultaneous VP_R.

TABLE I. Effect of Total Renal Venous Occlusion on Intrarenal Pressure.

	Intrarenal pressure		Blood pressure		
Dog No.	Before occlusion	During occlusion	Systolic	Diastolic	Heart rate
1	36	57	153	98	238
2	14	55	137	57	164
3	15	63	130	75	89
4	35	77		105	131
5	18	48		138	167
6	10	83		105	160
7	23	95	162	105	166
8	59	124	172	120	138
9	27	90	142	98	214
10	15	40	75	40	253
11	18	109		100	105
Mean	25	76			

Partial occlusion. The data for 5 dogs are shown in Fig. 1. When the renal vein is partially occluded, the IRP is not affected until the VP_R is elevated above the initial IRP. As the occlusion is further increased, thus increasing VP_R, IRP rises with the VP_R in a one-to-one relationship. When the occlusion is complete, the IRP is the same as the simultaneous VP_R as indicated in the figure.

The VP_R in total occlusion does not consistently rise, just as the IRP does not rise, to the simultaneous diastolic blood pressure. Records were obtained on this point in 4 dogs: in the first, when the VP_R with total occlusion was 78 mm Hg, the simultaneous diastolic pressure was 100, in the second the 2 pressures were 84 and 82, in the third 85 and 110 and in the fourth 98 and 115.

Discussion. When the venous outflow of an organ is completely blocked, the simultaneous venous pressure rises to arterial pressure (8,9). But in the present experiment, the venous outflow was not completely blocked since the capsular circulation remained intact. This collateral circulation, irregular though it

---

6. Swann, H. G., Montgomery, A. V., Davis, J. C., and Mickle, E. R., *J. Exp. Med.*, 1950, v92, 625.

7. Kubicek, W. G., Sedgwick, F. P., and Visscher, M. B., *Rev. Scient. Instr.*, 1941, v12, 101.

8. Flack, M., and Hill, L., *A Textbook of Physiology*, Edward Arnold, London, 1919, p225.

9. Duffield, F. A., *J. Physiol.*, 1934, v81, 283.

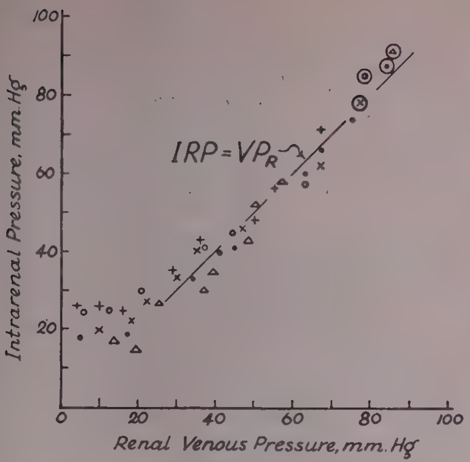


FIG. 1.

Relation of intrarenal pressure and renal venous pressure during venous occlusion. Individual records of 5 dogs. Encircled values are taken after complete occlusion.

is, undoubtedly drained away some blood when the main renal vein was occluded, and thus the renal vein pressure did not always rise to the simultaneous arterial pressure. When the VP_R is increased by partial occlusion to or above the control IRP, it causes the IRP to rise to the simultaneous VP_R. Gottschalk(5) has reported this likewise. The same general phenomenon was studied in arm veins by Ryder, *et al.*(10), who showed that the pressure in arm veins (when adjusted to heart level) is equal to the tissue pressure, and that when the arm veins are partially occluded with a cuff, the distal venous pressure at once rises just above the cuff pressure.

The close correspondence of IRP and VP_R (above the control IRP) furnishes confirmation of our hypothesis that the IRP measures the pressure within the renal venules(11). Furthermore, the fact that the IRP is not influenced until the VP_R rises above 25 mm Hg confirms our hypothesis that the pressure in these same vessels is also at about 25 mm. Moreover, because the IRP has been found to be so uniform through all the renal parenchyma(6), it is also presumed that this pressure is transmitted to all the tubes in the kidney. Hence, in order for fluids to flow through the various tubes, their pressures must be greater than the IRP or else they would collapse. Arteriolar and glomerular pressures are undoubtedly higher than the IRP, but all other renal tubes are thought to have a pressure of about 25 mm Hg: the peritubular capillaries and venules, the lymphatics and the entire uriniferous tubular system with its intracapsular space(11).

The characteristics of fluid flows through a system like that postulated are of considerable interest. In order to gain some insight into them, the behavior of the model illustrated in Fig. 2 was studied. Reservoir H delivered water into elastic tube E (actually "Penrose" tubing, I.D. 12.8 mm) and thence to outlet V, at which flow rates were measured. The elastic tube E was surrounded by the rigid, water-filled cylinder K, the pressure in which was controlled by reservoir T. The pressures in reservoir H and reservoir T will be designated by the symbols H and T respectively. (Air bubble C prevented the system from "bouncing".) The lateral pressures at A and B were also measured. At O, an adjustable, occluding tourniquet was placed so that the flow, and hence the pressure, at B could be readily varied after flows of water through the system had been established. When pressure T was imposed upon elastic tube E, little or no flow took place until pressure H exceeded pressure T. Regardless of the pressure at H, if it exceeded T, the pressure at A was just slightly greater than pressure

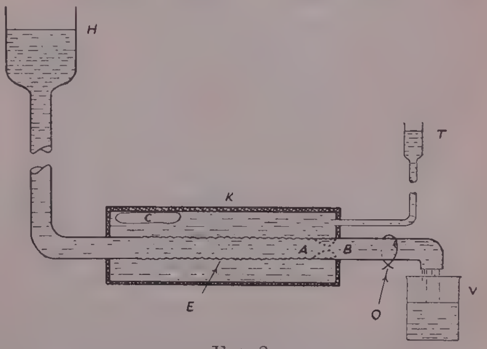


FIG. 2.

Model used for studying pressure and flow relations. See text for explanation.

---

10. Ryder, H. W., Molle, W. E., and Ferris, E. B., *J. Clin. Invest.*, 1944, v23, 333.

11. Montgomery, A. V., Davis, J. C., Prine, J. M., and Swann, H. G., *J. Exp. Med.*, 1950, v92, 637.

T, while the pressure at B, only a millimeter or two downstream, dropped almost to zero.

In this model, cylinder K is equivalent to the kidney and pressure T is equivalent to the renal tissue pressure, while reservoir H represents the hydrostatic pressure of the blood given by the heart. Furthermore, pressure T is imposed on the whole length of E until tube E emerges from cylinder K; at this point the pressure suddenly drops almost to zero.† Continuing the analogy, IRP is imposed on peritubular capillaries, venules, lymphatics and tubules until they emerge from the kidney. At this point, the pressure in them suddenly drops toward zero—actually, in the case of the veins, toward vena cava pressure. Now, renal vein pressure has been reported to be 11 mm Hg(12), or only a few millimeters above caval pressure. This is similar to taking a pressure at B. If in such experiments the catheters used to measure renal venous pressure were inserted all the way to A, then, it is postulated, the renal venous pressure would be found just to exceed IRP. The point of pressure change is undoubtedly somewhere above the epithelial lining of the pelvis. Here the pressure abruptly rises, it is thought, to 25 mm Hg.

When tourniquet O is tightened so that the pressure at B is elevated, no change in the rate of flow occurs until pressure B exceeds pressure T. Thus, if T is 25 mm Hg, pressures at B, induced by tightening tourniquet O, of 10 or 15 or 20 mm Hg do not slow the flow of water out of the system. It is only when the pressure at B is 25 mm or greater that flow is slowed. The same situation pertains in the kidney, for both Blake, *et al.*(13) and Selkurt, *et al.*(14) have demonstrated that renal blood flow is not appreciably influenced by renal vein pressures up to some 22 mm Hg. In the experiments of Blake, *et al.*(13), it was only when the renal vein pressure was raised to 40 mm Hg that renal blood flow was slowed. This fact furnishes good confirmation from independent sources of observation that the IRP is above 22 mm Hg. Similarly, if there were a pressure of 25 mm Hg in the renal tubules, one would not expect that urinary flow rate to change unless ureteral pressure exceeded 25 mm. This in fact has been demonstrated by Pilcher, *et al.* (15): in normal dogs, ureteral pressures of 22 mm Hg do not slow urine flow but 29 mm of pressure does. The relatively low value of 10 mm Hg, reported by Winton(16) in the same experiment, is thought due to the fact that he was studying such a relatively abnormal preparation, *viz.* the isolated perfused kidney(17,18).

It has been pointed out that the intrarenal pressure is exceptionally high for a tissue pressure(11). Furthermore, it has been shown that the venous pressure of subacute congestive heart failure, elevated though it is, does not exceed the exceptionally high IRP(19). It was therefore suggested(19) that the IRP is normally, as it were, "set high" so that random or pathological fluctuations in vena cava pressure do not influence renal blood flow or renal tubular function. Something akin to this has been done in the present experiment, in which elevations in the renal venous pressure exert no influence on IRP until they exceed it. Because vena cava pressure is so much smaller than IRP, it does not, as in the studies on the model reported here, and as in the experiments of Blake, *et al.*(13) and Selkurt, *et al.*(14), influence renal blood flow until it exceeds the exceptionally high renal tissue pressure. This situation permits the whole delicate interplay of tubular processes to continue at a relatively constant pressure.

---

† In the model, there can be perceived a constriction between A and B (indicated by dotted lines), the magnitude of which is directly proportional to the ratio of pressure A to pressure B.

12. Maxwell, M. H., Breed, E. S., and Schwartz, I. L., *J. Clin. Invest.*, 1950, v29, 342.

13. Blake, W. D., Wegria, R., Keating, R. P., and Ward, H. P., *Am. J. Physiol.*, 1949, v157, 1.

14. Selkurt, E. W., Hall, P. E., and Spencer, M. P., *Am. J. Physiol.*, 1949, v157, 40.

15. Pilcher, F., Bollman, J. L., and Mann, F. L., *J. Urol.*, 1937, v38, 202.

16. Winton, F. R., *Physiol. Rev.*, 1937, v17, 408.

17. Shannon, J. A., and Winton, F. R., *J. Physiol.*, 1940, v98, 97.

18. Selkurt, E. E., *Am. J. Physiol.*, 1945, v145, 376.

19. Prine, J. M., Mathis, L. R., and Swann, H. G., in press, 1951.

This theory presumes that hydrostatic pressure, either blood or tubular or interstitial, is held constant in the tubular sections of the nephron; these constant pressures are presumed to play no role in the process of absorption. Rather, the differential absorptive process is governed entirely by fluctuating oncotic pressures and cellular metabolic processes.

Summary. 1. When the renal vein is completely occluded, the intrarenal pressure increases about 3-fold, being elevated to the simultaneous pressure in the vein on the renal side of the occlusion. Usually this pressure is somewhat less than the simultaneous diastolic blood pressure. When the vein is partially occluded, intrarenal pressure does not change until the simultaneous pressure in the vein on the renal side of the occlusion rises to the original intrarenal pressure. At this point, as the occlusion is increased, the two pressures rise together and always have the same value. 2. The hypothesis is proposed that the pressure inside the renal peritubular capillaries, venules, uriniferous tubules and lymphatics is normally just above intrarenal pressure, *i.e.* at 25 mm Hg. This situation, it is postulated, permits the process of reabsorption to take place freed from the influence of random or pathological fluctuations in vena cava pressure. Oncotic pressures and cellular metabolic processes, therefore, are presumed to be the primary determinants of differential reabsorption.

Received March 21, 1951. P.S.E.B.M., 1951, v76.

Resistance Lowering Effect of Human Respiratory Tract Mucin.* (18630)

W. J. NUNGESTER, J. K. BOSCH, AND DARWIN ALONSO.
From the Department of Bacteriology, University of Michigan.

Although hog gastric mucin has been extensively used since 1932(1) to lower the resistance of laboratory animals to bacteria, the possible role of excessive mucus accumulations on the defense mechanisms of man has received little attention. Because of the commercial availability of hog gastric mucin, this material has been used almost exclusively as an aid in producing experimental infections. The senior author in unpublished work has used human respiratory tract mucin in a few trials with results quite similar to those obtained with gastric mucin. It seemed desirable to confirm and extend these earlier unpublished observations with human respiratory tract mucin since there are many clinical situations where excessive mucus could be significant in lowering the resistance of man.

The human mucin used in this study was obtained from sputum collected from patients with varying lung conditions (lung neoplasms, bronchiectasis, lung abscess, asthma, suppurative pneumonitis). The different samples of sputum were homogenized together in a CO_2 atmosphere by means of a Waring blendor. The mucin was then sterilized by heating at 80°C for ten minutes. Sterility of the mucin was controlled by culture on a blood agar plate.

Intra-bronchial inoculations in rats. A small vertical incision was made through the skin and muscles overlying the trachea to aid in passing a number 5 French ureter catheter into a main bronchus. Although the exposed trachea was not opened, a clear view was had of the descending catheter. In this manner, passage of the inoculum into the esophagus was avoided with certainty. After inoculation, the skin incision was closed with a single suture. Four series of experiments were done, each consisting of 5 groups of 5 rats each. A suspension of pneumococci, type I, was suspended in different menstrums and 0.2 cc was injected intra-bronchially into each animal. Each rat received an inoculum of 10^{-4}

---

* Aided by a grant from the U. S. Public Health Service.

1. Nungester, W. J., Wolf, A. A. and Jourdonais, L. F., PROC. SOC. EXP. BIOL. AND MED., 1932, v30, 120.

TABLE I. Effect of Human Respiratory Tract Mucin in Lowering Resistance of Lungs of Rats to Pneumococcus Pneumonia.

Inoculum	No. of animals Inoculated	Pneumonia involving lobe >50% %	20–50% %	<20% %	0% %
Pnc. I 10^{-4} ml in human mucin	20	60	20	20	
Pnc. I 10^{-4} ml in hog gastric mucin	20	80	20		
Pnc. I 10^{-4} ml in saline	20				100
Human mucin alone	20			100	
Hog gastric mucin alone	20			100	

ml of a culture having a plate count of approximately 10^8 per ml. After 48 hours the animals were sacrificed. Table I presents the results which clearly indicate that human mucus was approximately as effective as hog gastric mucin in lowering the resistance of the rat lung to pneumococcus pneumonia. In order to eliminate such factors as atelectasis from a consideration of the resistance lowering properties of human mucus, the intrabronchial experiments in rats were discontinued in favor of intraperitoneal inoculation of mice. It is generally accepted that the main effect of mucin in intraperitoneal infections involves the phagocytic cells and possibly the intracellular destruction of the bacteria(2,3). There is essentially no evidence that mucin increases the virulence of bacteria, a careless implication originally extended by the senior author.

Intraperitoneal inoculations in mice. A culture of a group C hemolytic streptococcus was used for the experiments, designed to evaluate the resistance lowering effect of human mucin and its fractions when injected intraperitoneally with the test organism. Four series of experiments were done. Each consisted of eleven groups of 5 mice. Three different dilutions of streptococci were suspended in human mucin so that the inoculum 0.25 ml contained 10^{-4}, 10^{-6}, or 10^{-8} of the original culture suspended in human or hog mucin or saline. Two control groups of mice received human respiratory mucin or hog gastric mucin alone. Table II presents the typical results of a test in this series of experiments. It is apparent that human mucin has a resistance lowering effect almost equal to that of hog gastric mucin. Morgan and King(5) isolated from crude gastric mucin a material identical with the human blood group A factor. Landy and Batson(6) have recently shown a correlation between the resistance lowering action of hog gastric mucin and its content of human blood group A antigen. This suggested a comparison of fractions of hog gastric and human mucin for resistance lowering properties and their content of blood group A antigen.

TABLE II. Resistance Lowering Properties of Human Respiratory Tract Mucin Tested by Intraperitoneal Inoculation of Mice.

Inoculum Streptococcus group C	Suspended in Human mucin	Hog gastric mucin	Saline
10^{-4}	5/5*	5/5	2/5
10^{-6}	5/5	5/5	0/5
10^{-8}	1/5	2/5	0/5

$$* \text{ Fraction} = \frac{\text{No. dead at 4 days}}{\text{No. inoculated}}.$$

Mucin solutions alone were not lethal.

Fractionation procedures. The fractions of hog gastric mucin† were obtained essentially by one of the methods of Morgan and King (5). This consists of extraction of the material with 90% phenol, centrifuging and then treatment of the supernate with ethanol to a given concentration. The precipitate formed at this ethanol concentration is centrifuged, washed

---

2. Nungester, W. J., Jourdonais, L. F., and Wolf. A. A., *J. Inf. Dis.*, 1936, v59, 11.

3. McLeod, C., *Am. J. Hyg.*, Sec. B., 1941, v34, 41.

4. Olitzki, L., *Bact. Rev.*, 1948, v12, 149.

5. Morgan, W. T. J. and King, H. K., *Bioch. J.*, 1943, v37, 640.

6. Landy, M. and Batson, C., *J. Immunol.*, 1949, v62, 4.

† Granular mucin type 170W—Wilson Laboratories.

TABLE III. Resistance Lowering Properties of Hog and Human Mucin and Fractions Tested by Intraperitoneal Inoculation of Mice.

Inoculum Streptococcus group C, ml	Suspended in						
	Hog gastric mucin, crude	A_2 from hog mucin	Human mucin, crude	N_1 from human	N_2 from human	N_3 from human	Saline
10^{-6}	5/5*	5/5	5/5	0/5	—	5/5	0/5
10^{-7}	4/5	5/5	4/5	0/5	3/5	1/5	0/5
10^{-8}	0/5	2/5	1/5	0/5	3/5	0/5	0/5

* Fraction = No. dead at 4 days/No. inoculated. Human blood group A inhibiting titers A_2, $<.001\ \gamma$; N_1, $.02\ \gamma$; N_2, $.09\ \gamma$; N_3, $.37\ \gamma$. Mucin and fractions alone were not lethal.

TABLE IV. Resistance Lowering Properties of Human Respiratory Tract Mucin and Fractions Tested by Intraperitoneal Inoculation of Mice.

Inoculum Streptococcus group C, ml	Suspended in					
	Crude mucin	M_1	M_2	M_3	M_4	Saline
10^{-6}	5/5*	5/5	5/5	5/5	3/5	0/5
10^{-7}	4/5	5/5	4/5	3/5	1/5	0/5
10^{-8}	3/5	3/5	1/5	2/5	0/5	1/5

* Fraction = No. dead at 4 days/No. inoculated. Human blood group A inhibiting titers M_1, $.0025\ \gamma$; M_2, $.02\ \gamma$; M_3, $.32\ \gamma$; M_4, $5.0\ \gamma$. Mucin and fractions alone were not lethal.

and dried from the frozen state. The supernate from this latter step is adjusted to a higher ethanol concentration and the resulting precipitate recovered and treated as above. The commercial hog gastric mucin was extracted after first homogenizing with the 90% phenol in a Waring blendor. The human mucin material, after sterilization with heat, as described above, was dried in vacuum and then suspended in 90% phenol as done with the hog gastric mucin. Each fraction obtained from both sources of material was, after washing with alcohol (95% ethanol), dialyzed against distilled water. The dialysis was carried out for 72 hours, water being changed every 24 hours. In the "N" series of fractions from human mucin, the amount of material obtained on adjustment of the supernatant to 10% ethanol was so small that in the subsequent series of fractions, series "M," the second fraction was obtained by adjustment of the supernatant to 33% ethanol. Two other fractions, besides that insoluble in 90% phenol and the "M"-2 fraction, were obtained by adjustment of the respective supernates to 55% and 75% ethanol respectively. These fractions were tested for resistance lowering effect by injecting 0.25 ml containing 10^{-6}, 10^{-7}, or 10^{-8} ml of the test culture intraperitoneally. The experiment included inoculations of organisms suspended in saline, 5% hog gastric mucin "A" fraction, 3% human mucin or fractions N_1, N_2, and N_3. Hog gastric mucin and human mucin alone were included as controls.

Table III records the results of these experiments. It will be noted that fraction N_3 of human mucin and A_2 of hog material were particularly effective in lowering resistance. The virulence enhancing factors in hog gastric mucin according to Morgan and King(5), Landy and Batson(6), are characterized by a strong blocking action of anti-human blood antigen A. Hemagglutination inhibition tests similar to the one described by Landy and Batson(6) were carried out on hog gastric mucin, human mucin, and their fractions. There was at least 300 times less blood group A substance in the N_3 fractions than in the A_2 fractions; yet both were active in lowering resistance. Also, the N_1 fraction with ten times more A substance than N_3 was devoid of resistance lowering properties, while N_3 material was active in this respect.

Table IV presents the results of tests on resistance lowering properties of the fractions. Fractions M_1, M_2, and M_3 were particularly active in lowering resistance. Again, there was no correlation between iso-hemagglutination inhibition and resistance lowering properties of the mucin fraction.

Summary. 1. Human respiratory tract mucin possesses a resistance lowering factor similar in activity to that of hog gastric mucin. This effect was shown by intrabronchial inoculation of rats with pneumococci suspended in saline or the two types of mucin. It was also shown by intraperitoneal inoculation of mice with streptococci suspended in saline, in two types of mucin and in fractions thereof. 2. Evidence against resistance lowering properties of mucin depending on the presence of blood group A antigen was obtained by fractionating human respiratory tract mucin and testing the fractions for blood group A antigen and the resistance lowering properties of the fractions.

Received March 22, 1951. P.S.E.B.M., 1951, v76.

Tissue Distribution, Accumulation and Elimination of the Isomers of Benzene Hexachloride. (18631)

BERNARD DAVIDOW AND JOHN P. FRAWLEY.* (Introduced by O. Garth Fitzhugh)

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D.C.

One of the properties of benzene hexachloride which makes it an effective agricultural insecticide is the relatively persistent action of residues of this material remaining after spraying or dusting. However, this property may give rise to a consumer hazard through the ingestion of foodstuffs contaminated by these residues. In order to evaluate the potential consumer hazard acute(1) and chronic (2) toxicity experiments have been conducted. It was noted that in single oral doses the toxicity of the isomers decreased in the order of gamma, alpha, delta and beta. However, in the chronic experiments it was observed that the beta isomer was the most toxic followed by alpha, gamma and delta. An explanation of this phenomenon was sought by investigating the tissue distribution, the relative degree of accumulation after feeding diets containing the various isomers, and the rate of elimination of the isomers from the fat after removal of the benzene hexachloride from the diet. Early work from this laboratory on the distribution of the gamma isomer indicated that it might be stored preferentially in the fat(3).

Method. Rats used in this study were from our stock of Osborne-Mendel strain and they were fed *ad libitum* a diet consisting of ground commercial laboratory chow to which had been added the various isomers of benzene hexachloride dissolved in a small quantity of corn oil. The analytical method employed was that of Davidow and Woodard which depends upon the conversion of the benzene hexachloride to 1,2,4-trichlorobenzene and the determination of the latter by its characteristic ultraviolet spectrum(4). To determine the tissue distribution of benzene hexachloride, weanling rats were placed on diets containing 800 p.p.m. of the alpha, gamma and delta isomers, and 100 p.p.m. of the beta isomer. Feeding of these diets was continued for approximately 20 months after which time the rats were sacrificed and the tissues removed. Dogs weighing from 11 to 16 kg were given gelatin capsules of corn oil solutions containing 5% of the alpha isomer, 2% of the gamma isomer, or 10% of the delta isomer. Due to the relative insolubility of the beta isomer in corn oil it was incorporated into ground commercial laboratory diet at a level of 0.2%. The dosages of the various isomers fed the dogs were selected so as to obtain an approximately equivalent degree of severe toxicity. For the accumulation study, rats ranging in weight from 140 to 200 g were fed diets containing 100 p.p.m. of the individual isomers of benzene hexachloride. At two-week intervals 2 male and 2 female rats were sacrificed for each of the diets. The abdominal fat from each rat was removed and analyzed for benzene hexachloride. For the elimination study, weanling rats ranging in weight from 50 to 80 g were placed on a diet containing 100 p.p.m. of the individual isomers of benzene hexachloride for a period of 6 weeks in order to obtain maximum storage. At the end of this time the diets were replaced by control diets. Two female and 2 male rats on each of the diets were sacrificed at the end of the 6-week storage period, and a similar number at weekly intervals thereafter. The abdominal fat was removed from the animals and analyzed for benzene hexachloride.

---

* Portions of this paper are excerpts from a thesis submitted by John P. Frawley in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Georgetown University.

1. Woodward, G., and Hagan, E. C., *Fed. Proc.*, 1947, v6, 386.

2. Fitzhugh, O. G., Nelson, A. A., and Frawley, J. P., *J. Pharm. and Exp. Therap.*, 1950, v100, 59.

3. Laug, E. P., *J. Pharm. and Exp. Therap.*, 1948, v93, 277.

4. Davidow, B., and Woodard, G., *J. Assoc. Off. Agri. Chem.*, 1949, v32, 751.

Results. If benzene hexachloride had been stored in the tissue as the 1,2,4-trichlorobenzene a chromatographed hexane solution of the tissues would have exhibited the characteristic absorption curve of the 1,2,4-trichlorobenzene(5). An examination of the absorption spectra showed that this was not the case. However, when the material in the hexane solution was refluxed with alkali it then exhibited the characteristic absorption curve of 1,2,4-trichlorobenzene, thus indicating that these isomers of benzene hexachloride were stored unchanged in the adipose tissue.

Tissue distribution. Analyses of tissues from 2 female rats on each diet (Table I) indicated that the greatest storage was to be found in the fat tissue; brain, kidney, liver and muscle showed the presence of some benzene hexachloride, but considerably less. The tissue distribution data for dogs are summarized in Table II. The dosages for the dogs fed the beta isomer were calculated from food consumption and average weight data. This data shows that the isomers of benzene hexachloride are preferentially stored in the adipose tissue of dogs. The alpha and beta isomers are stored to a lesser degree in liver, kidneys and adrenals, while the gamma isomer is stored to some extent in the kidneys and the adrenals. The liver, kidneys and adrenals of dogs fed the delta isomer show no detectable storage.

TABLE I. Tissue Distribution of Isomers of Benzene Hexachloride in Female Rats After 20 Months' Feeding.*

	—— 800 p.p.m. diet level ——			100 p.p.m. diet level
	Alpha (p.p.m.)	Gamma (p.p.m.)	Delta (p.p.m.)	Beta (p.p.m.)
Fat	3500	440	550	1900
Brain	230	73	75	130
Kidney	180	75	97	150
Liver	120	Neg.	20	20
Muscle	75	20	38	75

* Each figure represents average storage for 2 animals.

Accumulation. The results of adipose tissue analyses on rats receiving benzene hexachloride in their diet and the striking differences in storage among the isomers can be seen in Fig. 1. It can also be seen that the concentrations of the isomers in the fat of female rats are generally higher than in the fat of males. It will be noted that the storage concentration of the isomers, with the possible exception of beta isomer, tends to level off between the fourth and sixth week and that no significant increase in accumulation appears after this point.

Elimination. The results of the analyses conducted on the abdominal fat of rats fed benzene hexachloride in their diet are best illustrated by Fig. 2. This figure clearly shows the rapid elimination of the alpha, gamma and delta isomers, and the persistent nature of the beta. In the case of the female rats fed the beta isomer, small amounts to the extent of 40 p.p.m. are still present after 14 weeks' feeding of a control diet.

Discussion. Although the number of dogs used in the distribution study is small, it would appear that there are certain differences between rats and dogs in degree of storage in

---

5. Davidow, B., *J. Assoc. Off. Agri. Chem.*, 1950, v33, 130.

TABLE II. Tissue Distribution of the Isomers of Benzene Hexachloride in Dogs.

Isomer	No. of dog	Dose, mg/kg	Weeks on exp.	Tissue distribution,* p.p.m.			
				Fat	Liver	Kidney	Adrenals
Alpha	1	60	12	3870	1520	215	2600
Beta	2	24, 30	19, 20	8825	1100	50	505
Gamma	4	10, 15	2–7	466	Neg.	140	372
Delta	2	120	14, 20	550	Neg.	Neg.	Neg.

* Each figure represents average storage.

the various organs. For example, in the case of the alpha and beta isomers, the relation between storage in livers and kidneys is reversed for the 2 species. Another species difference was observed in the sensitivity of dogs to the gamma isomer, as is noted in Table II. However, this comparison is complicated by the fact that the dogs received more toxic doses of the isomers than the rats and died (2-20 weeks) before having had opportunity

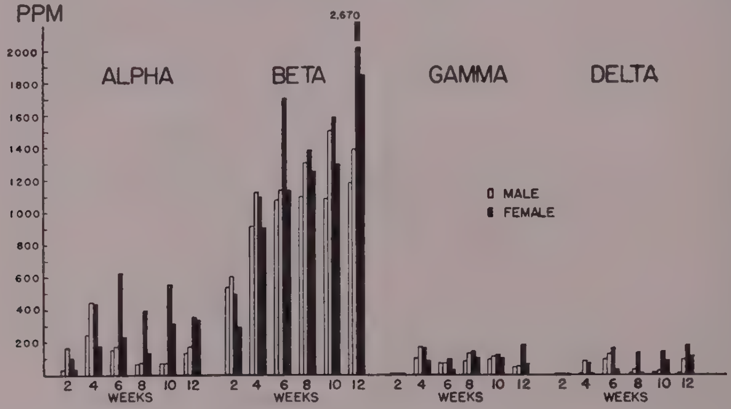


FIG. 1.

Accumulation of the isomers of benzene hexachloride in fat tissue of rats fed 100 p.p.m.

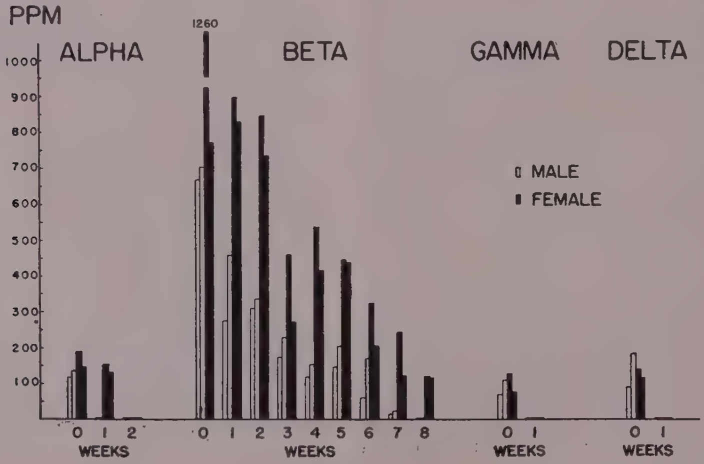


FIG. 2.

Disappearance of the isomers of benzene hexachloride from fat tissue of rats following the removal of the toxicant from the diet.

to develop a characteristic picture of long-term effects. Certainly, other factors than mere storage in the fat determine the toxicity of compounds ingested over long periods of time. However, we believe that here we have an example of a parallelism between gross signs of chronic toxicity and tissue accumulation.

Summary. 1. The alpha, beta, gamma and delta isomers of benzene hexachloride are preferentially stored in the adipose tissue of rats and dogs. 2. Some storage of benzene hexachloride is found in kidneys, brain, liver and muscle tissue of rats. 3. Dogs show considerable storage of the alpha, beta and gamma isomers in the adrenals. 4. The degree of accumulation in the adipose tissue bears a direct relationship to the chronic toxicity of the isomers. 5. The relative rates of accumulation in rat adipose tissue show that the approximate maximum storage is accomplished within 6 weeks. 6. Upon removal of the benzene hexachloride diet, alpha, gamma and delta isomers disappear from rat fat depots within 3 weeks; the beta still persists in small amounts after 14 weeks.

Received February 19, 1951. P.S.E.B.M., 1951, v76.

Amino Acid Composition of Dentin Protein.* (18632)

F. L. Losee,[†] B. A. Neidig, and W. C. Hess.

From the Department of Biological Chemistry, Georgetown Medical and Dental Schools, Washington, D.C.

The nature of the protein in the enamel of human teeth has been studied by Losee and Hess(1), Losee, Neidig, and Hess(2), and Block, Horwitt, and Bolling(3). From these investigations it appears that enamel protein is an eukeratin according to the criteria of Block. Block *et al.*(3) have also investigated the nature of human dentin protein by the technic of paper chromatography applied to the hydrolysate of dentin protein prepared by us and, although their results were qualitative, they have classified it as a collagen. Using quantitative colorimetric, chromatographic, and microbiological methods we have determined a number of the amino acids present in dentin protein.

Experimental. The protein was prepared from dentin ground from the fully erupted, permanent, non-carious first and second human molars used for the isolation of enamel protein(1). The teeth were cut at the cementoenamel junction and only the crown was employed. The ground dentin was dried in a desiccator, over anhydrous calcium chloride, for 48 hours and then demineralized by the method used to prepare enamel protein(1). The isolated protein was dried in the same manner as the original dentin. The yield of dentin protein obtained in several separate experiments was between 10 and 11 percent based upon the dry weight of the dentin. The sample of dentin protein used for the various determinations of the amino acids contained 2.06% ash and 16.09% nitrogen calculated upon the weight of the desiccator dried sample. Three methods of analysis were employed: colorimetric procedures applied directly to an acid hydrolysate of the protein, colorimetric procedures upon eluates resulting from chromatographic separation of the amino acids from the hydrolysate

---

* These studies were substantially aided by a contract between the Office of Naval Research, Department of the Navy and Georgetown University (NR 181-817) and also by a grant from the Sugar Research Foundation.

[†] LCDR (DC) USN, Naval Dental School, Bethesda, Md.

1. Losee, F. L., and Hess, W. C., *J. Dent. Res.*, 1949, v28, 512.

2. Losee, F. L., Neidig, B. A., and Hess, W. C., *J. Dent. Res.*, 1950, v29, 815.

3. Block, R. J., Horwitt, M. K., and Bolling, D., *J. Dent. Res.*, 1949, v28, 518.

by the method of Bergdoll and Doty(4) as modified by us(2) using a mixture of Lloyd's reagent and Hyflo Super-Cel and also microbiological assay. The protein was hydrolyzed by heating with 20% hydrochloric acid for 6 hours in a wax bath maintained at 125°. The hydrolysate was evaporated to dryness under reduced pressure to remove the hydrochloric acid and made to a definite volume with distilled water.

Phenylalanine, cystine, methionine, and tryptophane were determined by the method of Hess and Sullivan(5-7). For the estimation of hydroxyproline the procedure of McFarlane and Guest(8) was employed. After the determinations by this method had been made the modification of Neuman and Logan (9) appeared and it was applied to one hydrolysate with the same result as originally obtained. Histidine, arginine, and lysine, the basic amino acids, were determined by the procedures of Macpherson(10), Thomas, Ingalls, and Luck(11), and Bergdoll and Doty(4) respectively. The periodic acid methods of Nicolet and Shinn(12) and Shinn and Nicolet(13) for the determination of serine and threonine were employed. The value for serine has been corrected for the presence of 1.1% hydroxylysine based upon the value obtained in collagen by Rees(14). Glycine and alanine were estimated by the methods of Alexander, Landwehr, and Seligman(15) and Alexander and Seligman(16) respectively. Tyrosine was determined by the method of Folin and Marenzi(17). The microbiological assays for arginine, histidine, and lysine were carried out at the National Institutes of Health by the methods of Stokes, Gunness, Dwyer, and Caswell(18). The results are given in Table I together with a comparison of some literature values upon gelatin and collagen. The values are based upon the weight of the desiccator dried sample and are not corrected for moisture or ash. Each value represents the average of at least duplicate determinations upon two different hydrolysates.

Discussion. The data indicate that the 13 amino acids thus far determined in dentin protein conform, in general, to the pattern of these same amino acids in collagen and gelatin. In addition to the literature values cited in Table I, which were selected because they represent series of determinations upon the same proteins by the several investigators, other values for individual amino acids may be mentioned. Macpherson(10) found 8.57% arginine in collagen by colorimetric methods and 4.47% lysine determined by the difference between the total basic nitrogen and the nitrogen of the combined arginine and histidine. Salo(22) found that the arginine content of collagens from various sources varied from 8.61 to 9.55% and the histidine content from 0.70 to 1.09%.

The large amount of hydroxyproline, 13%, is in accord with the value of 14.4% found by Bergmann(23) in a sample of gelatin by

---

4. Bergdoll, M. S., and Doty, D. M., *Ind. and Eng. Chem., Anal. Ed.*, 1946, v18, 600.

5. Hess, W. C., and Sullivan, M. X., *Arch. Biochem.*, 1944, v5, 165.

6. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, 1943, v151, 635.

7. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, 1944, v155, 441.

8. McFarlane, W. D., and Guest, G. H., *Canad. J. Res. B.*, 1939, v17, 139.

9. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.

10. Macpherson, H. T., *Biochem. J.*, 1942, v36, 49.

11. Thomas, L. E., Ingalls, J. K., and Luck, J. M., *J. Biol. Chem.*, 1939, v129, 263.

12. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, 1941, v139, 687.

13. Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, 1941, v138, 91.

14. Rees, M. W., *Biochem. J.*, 1946, v40, 632.

15. Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, 1945, v160, 52.

16. Alexander, B., and Seligman, A. M., *J. Biol. Chem.*, 1945, v159, 11.

17. Folin, O., and Marenzi, H. D., *J. Biol. Chem.*, 1929, v83, 89.

18. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, v160, 35.

19. Bowes, J. H., and Kenten, R. H., *Biochem. J.*, 1948, v43, 358.

20. Graham, C. E., Waitkoff, H. K., and Hier, S. W., *J. Biol. Chem.*, 1949, v177, 529.

21. Neuman, R. E., *Arch. Biochem.*, 1949, v24, 289.

22. Salo, T. P., *Arch. Biochem.*, 1949, v24, 25.

23. Bergmann, M., *J. Biol. Chem.*, 1935, v110, 471.

TABLE I. Amino Acid Content of Dentin Protein.

	Method of determination			Literature values			
Amino acid	Color, %	Bergdoll and Doty chromat., %	Micro-biol., %	Bowes and Kenten(19) collagen, %	Graham *et al.*(20) Gelatin, %	Collagen, %	Neuman(21) collegan, %
Lysine		2.4	3.34	4.5	4.1	3.5	4.9 – 5.7
Arginine	7.4	7.5	6.91	8.8	8.0	7.0	8.6 – 9.4
Histidine	.7	.8	.8	.8	.8	.9	.56– .94
Hydroxyproline	12.9*	13.1		14.0			
Phenylalanine	1.9			4.2	2.2	2.5	2.3 – 2.6
Methionine	.5			.8	1.0	.9	.69– 2.3
Cystine	.1			.0	.07	.3	.05– .12
Tryptophan	.0			.0			.0
Serine	3.1			3.4			2.8 – 3.2
Threonine	1.9			2.4	1.9	2.5	
Glycine	19.0			26.2			23.3 –29
Alanine	8.5			9.5			8.7 –10
Tyrosine	1.1			1.4	.44	.9	.86– 1.06
Nitrogen	16.09			18.6	17.6	16.9	17.8 –18.4

* 13.0 by the method of Neuman and Logan(9).

isolation methods. Similar values were obtained by McFarlane and Guest(8) and more recently by Neuman and Logan(9), although no values for this amino acid in collagen itself were found in the literature. No other protein thus far analyzed has shown this high content of hydroxyproline.

Our values are upon desiccator dried samples of dentin protein, the literature values are, in most cases, upon moisture free, ash free proteins. We have observed definite signs of decomposition on drying dentin protein in an oven at 105° and have preferred to analyze the desiccator dried samples. The nitrogen value of 16.07%, uncorrected for moisture and ash, is lower than the literature values upon collagen and gelatin which are corrected for moisture and ash and range from 16.9 to 18.6%. These differences may account for our finding of 19% glycine which is lower than the cited values but, on the other hand, our value for tyrosine of 1.1% is higher than the literature values which range from 0.44 to 1.06% in gelatin and in collagen. However it is apparent that in general the pattern of amino acid composition, particularly the high concentration of hydroxyproline and glycine and also the absence of tryptophan, resembles that of collagen.

Summary. Protein prepared from the dentin removed from the crowns of noncarious, fully erupted, permanent, first and second human molars has been analyzed for lysine, arginine, histidine, hydroxyproline, phenylalanine, methionine, cystine, tryptophan, serine, threonine, glycine, alanine, and tyrosine. The amounts found of many of these 13 amino acids, particularly the values for glycine, hydroxyproline, and the absence of tryptophan suggest that dentin protein resembles collagen.

Received July 28, 1950. P.S.E.B.M., 1951, v76.

Metabolite-Resistance and Virulence of Smooth *Brucella* Variants Isolated after Prolonged Cultivation. (18633)

ROBERT J. GOODLOW, WERNER BRAUN, AND LEONARD A. MIKA.
From Camp Detrick, Frederick, Md.

Recent studies have shown that population changes in synthetic broth cultures of smooth *Brucella abortus*, 6232, are affected by the concentration of alanine accumulating in the culture medium as a product of normal metabolism(1). The addition of D-alanine to synthetic broth cultures(2) of smooth cells was found to inhibit the growth of the original inoculum and to accelerate the establishment of alanine-resistant nonsmooth (*e.g.*, rough, mucoid) variants or mutants(3). Recently it was observed that after prolonged (30 days) incubation (37°C) of originally smooth cultures in which nonsmooth types had established themselves, a renewed increase in viable cell counts occurred coincidental with the progressive establishment of a smooth type that has been labeled S′. By acriflavine test (4) and general consistency, colonies of this smooth type appeared identical with the original smooth inoculum, but when cell suspensions of the two were mixed and plated on "2-1" agar(5) the S′ type gave rise to colonies distinctly smaller than those of the original S type. After 60 days of incubation another smooth variant appeared and rapidly increased in numbers. Colonies of this type, labelled "dwarf smooth" or dS were extremely small, but morphologically, tinctorially, and antigenically (by agglutinin absorption and acriflavine test) the individual cells were indistinguishable from the original S or the S′ type.

In order to determine further the extent of similarities between these various S types, studies of their relative virulence for guinea pigs were undertaken. Since the establishment of nonsmooth variants in similar cultures was shown previously to be due to their relative resistance to the inhibitory effects of the metabolite alanine, the alanine resistance of the different smooth types was determined. Susceptibility of the original S type to the toxicity of alanine has been well established (1,2). Demonstration of increased alanine resistance or altered virulence of the derived smooth types (S′ and dS) that established themselves upon prolonged cultivation would indicate that the observed population changes do not represent reversions to the original S type but involve mutants that merely simulate many characteristics of the smooth stem type.

Results. Nine respective groups of 10 guinea pigs were infected subcutaneously with graded doses of the original S, the S′, or the dS strains of *Br. abortus*, 6232, each group receiving a particular dose of the designated strain (Table I). Animals were sacrificed 5 weeks after infection, and the regional lymph nodes, spleen, and liver were cultured on modified tryptose agar plates. Results of these virulence titrations are summarized in the table. Probit analysis of these data indicates that the virulence of the S′ and dS mutants is at least comparable to that of the original smooth type.

In order to determine the relative resistance of the original S, the S′, and the dS types to the inhibitory effects of alanine, a series of flasks was prepared, each containing 100 ml of synthetic medium and to each flask were added 100 μg or 500 μg DL-alanine per ml. These media were inoculated with 1 ml of turbidimetrically adjusted saline suspensions of S, S′, or dS. The cultures were incubated at 37°C, and the number of viable cells (plate count) was determined at regular intervals throughout the period of observation. The results, shown in Fig. 1, revealed significant differences in the resistance of the three S types to the inhibitory effects of 500 μg of

---

1. Goodlow, R. J., Mika, L. A., and Braun, W., *J. Bact.*, 1950, v60, 291.
2. Goodlow, R. J., Braun, W., and Mika, L. A , *Arch. Biochem.*, 1951, v30, 402.
3. Braun, W., *Bact. Revs.*, 1947, v11, 75.
4. Braun, W., and Bonestell, A. E., *Am. J. Vet. Res.*, 1947, v8, 386.
5. Braun, W., *In "Brucellosis" AAAS Symposia*, 1950, v10, 26.

TABLE I. Virulence for Guinea Pigs of S, S′, and dS Types of *Brucella abortus*, 6232.

Strain	Avg No. organisms inj. per animal	Infection ratio (infected over total)	Estimate of ID_{50}	95% confidence limits	
				Lower	Upper
S	8.5	5/10			
	39.0	5/10	9.9	1	96
	78.0	9/10			
S′	8.4	7/10			
	40.2	9/10	1.3	.02	95
	80.4	9/9			
dS	8.4	6/10			
	39.6	5/10	11.7	1	133
	79.2	9/10			

alanine per ml. Similar but not as marked differences were noted with 100 μg DL-alanine per ml. Thus, despite similarity in many properties, the S, S′, and dS types proved to be dissimilar in their alanine resistance.

Discussion. These results demonstrate that in the course of progressive population changes in a closed system, mutants may establish themselves that simulate the parent type in many characteristics but differ from it in those properties responsible for increased selective value in the presence of inhibitory metabolites. It was shown that the 3 smooth types, isolated at different times in the culture's history, were similar in virulence, antigenic properties, and many other characteristics, yet differed significantly in their resistance to alanine, a compound which is known to accumulate in these cultures as an end product of metabolism. This difference in alanine resistance permitted the S′ and dS mutants to establish themselves at periods when alanine levels of the cultures were high and when the S parent type would have failed to find a favorable environment. Thus, what superficially appeared to involve reverse population changes, or the establishment of reverse mutants, actually involved the establishment of novel mutants with phenotypes similar to that of the original smooth cells. Many past observations recorded as reverse changes(3) may represent other examples of progressive mutational changes. Similar results have been noted recently in studies involving phenotypic changes from streptomycin-dependence to streptomycin-resistance in *E. coli*(6). Such observations raise the questions whether true reverse mutational changes occur at all and if so at what frequency. In order to determine the occurrence of true reverse mutants, an environment free of inhibitory metabolites would be required, at least for *Brucella*, in which the role of metabolites in the determination of selective values now has been recognized.

It is noteworthy that the progressive establishment of novel smooth types in cultures of

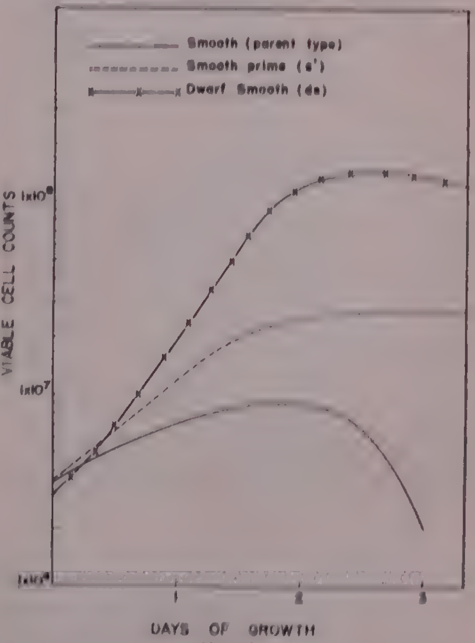


FIG. 1.

Viable cells (plate counts) of S, S′, and dS on various days in synthetic medium containing 500 μg of DL-alanine per ml.

---

6. Demerec, M., *Trans. N. Y. Acad. Sci.*, 1950, v12, 186.

Brucella after prolonged cultivation has involved reversions to virulence in mutants originating from relatively avirulent nonsmooth types. Thus, it would appear that in most instances the potentiality for virulence is not really lost* but its manifestation is merely suppressed and may be reestablished by various types of mutational change. As frequently suspected in the past(3), this ability of relatively avirulent cells to yield new mutants with high virulence, capable of establishing themselves under proper environmental conditions, may be of significance to problems of pathogenesis and epidemiology.

Summary. Following prolonged incubation of originally smooth *Brucella abortus* broth cultures, relatively avirulent nonsmooth variants established themselves but were eventually replaced by a smooth type, labelled S', in turn replaced later by still another smooth type, labelled dS. These three smooth types were found to be similar in many characteristics, including virulence, but differed significantly in their resistance to alanine, an inhibitory metabolite which accumulates in the culture fluid. These differences in alanine resistance permitted the S' and dS mutants to establish themselves at periods when alanine levels of the cultures were high. Therefore, what superficially appeared to be an establishment of reverse mutants actually involved the establishment of novel mutants which simulated the original smooth type in many characteristics.

---

* Strain 19 of *Br. abortus* seems to be one exception.

Received March 27, 1951. P.S.E.B.M., 1951, v76.

Influence of Alpha-Naphthyl Thiourea on Gastric Evacuation.* (18634)

Sally H. Dieke. (Introduced by C. P. Richter)

From the Psychobiological Laboratory, Johns Hopkins Medical School, Baltimore, Md.

In the course of earlier experiments performed in this laboratory(1), it was noted that the stomachs of rats dying from acute poisoning with alpha-naphthyl thiourea (ANTU) were frequently hugely distended with food, and since the deaths occurred some hours after the rats had eaten, this suggested the possibility that the ANTU had in some way interfered with the normal process of gastric evacuation. The experiments described in the present paper were, therefore, planned to investigate the possible effect of various doses of ANTU on gastric emptying time.

Methods. The experiments were performed with adult albino rats, both male and female, of the Wistar strain or a strain derived from it. The acute median lethal dose (LD_{50}) of ANTU, as administered to these rats, was roughly 5 mg kg of body weight; doses of 2 mg kg killed none, while 10 mg kg was almost invariably fatal within 7 to 17 hours (2). The rats were trained to eat a sizable meal in one hour or less; ANTU suspended in olive oil was then administered by intraperitoneal injection, and the weights of the stomach contents at varying intervals afterwards were then compared with those of control rats that received only olive oil. In one experiment banthine† was given to a group of rats to compare the effect of this drug with that of ANTU in delaying gastric evacuation. Data on a few rats receiving epinephrine‡ were also obtained. The rats were trained to eat a substantial amount of food in a short time by offering food for only a stated 2-hour

---

* Carried out under a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1. Richter, C. P., *J.A.M.A.*, 1945, v129, 927.

2. Unpublished data from this laboratory.

† Diethylaminoethyl xanthine-9-carboxylate methobromide, provided through the courtesy of Dr. Irwin C. Winter of G. D. Searle & Co.

‡ 1:500 suspension in peanut oil, from Abbott Laboratories.

period of each day during a week or 10 days' training period. Water was available at all times. The food used was our regular stock diet.§ Frequently a rat did not eat on the first or even the second day, and occasionally rats ate so little as to die of inanition, but by the end of a week the majority were avid eaters, waiting for the food to be put in their cages and consuming it rapidly. In one typical group of 24 rats on this regimen, 1 died on the 7th day, while the food intake of the 23 survivors on the 10th day averaged 12.2 g, ranging from 7.5 to 29.3 g. These rats thus ate in 2 hours about as much food as they normally would eat in 24 hours. The average loss of body weight during the training period was 17 g, or 4.7% of the average initial body weight (363 g).

On the day of poisoning, the drinking water was removed and the rats were offered food that they were allowed to eat for one hour. Occasionally rats stopped eating before the hour was up. Immediately after this meal, (or just before, in one experiment) the rats received a dose of ANTU, banthine or epinephrine. The doses were graduated to the rats' body weights, and were given by intraperitoneal injection rather than by mouth to avoid any direct effect on the gastro-intestinal tract. Control rats received only olive oil, also by intraperitoneal injection. Groups of drugged and control rats were always carried through in parallel on the same days. At hourly intervals thereafter, the rats were sacrificed (by crushing the cervical spine with pliers) and autopsied. The stomach was exposed, separated from adjacent organs, tied off with silk thread at the cardiac and pyloric sphincters, and weighed; it was then split lengthwise, emptied and reweighed, the difference giving the weight of the contents. In those experiments in which dry weights were also determined, the contents were placed in individual evaporating dishes and dried in an oven at 110°C to constant weight loss, together with control dishes containing equivalent amounts of food.

Data on 137 rats poisoned with ANTU, 10 rats receiving banthine, 13 rats receiving epinephrine, and 71 control rats are included here. The observations cover the 24 hours subsequent to poisoning. ANTU was given at 3 dosage levels: 15 rats received 2 mg/kg, a sublethal dose; 52 had 5 mg/kg, from which dose approximately 50% of the rats might be expected to survive, but which customarily produces no casualties in the first 18 to 20 hours(2); and 70 received 10 mg/kg, 16 just before and 54 just after feeding. Casualties from this last dose are usually 100%, with the rats beginning to die at 7 hours, and in fact the data between 7 and 16½ hours were obtained at death, and no data for intervals longer than this could be got.

Results. Fig. 1 shows that ANTU caused a delay in gastric evacuation, the degree of which depended on the amount of ANTU administered. Average values of the ratio of stomach contents to food eaten are plotted against the time elapsed after feeding ended. It will be seen that for the control rats the ratio of stomach contents to food eaten ranged from 1.30 to 1.60 directly after eating¶. The ratio then decreased gradually for the controls, as the stomach emptied, reaching an average 0.39 at 12 hours and becoming 0.02 at 24 hours.‖ In marked contrast to the controls, the values obtained in rats receiving 10 mg/kg of ANTU never fell below 1.29 and at 16½ hours a value of 1.75 was obtained, indicating minimal discharge of food from the stomach. At all times these values were well above the highest individual values obtained with control rats.

The ratios of stomach contents to food

---

§ Graham flour 72.5%, crude casein 10.0%, skim milk powder 10.0%, butter 5.0%, calcium carbonate 1.5% and sodium chloride 1.0%.

¶ This is above 1.00 on account of the oral and gastric secretions—see below for dry weights.

‖ According to x-ray studies on the rat's gastrointestinal tract(3), a barium meal is normally evacuated from the stomach in about 2 hours, with a range of 1 to 3 hours. Since such a meal is liquid and of high specific gravity, it might be expected to leave the stomach more quickly than the solid food eaten by the rats in the present experiment.

3. Gershon-Cohen, J., and Shay, H., in *The Rat in Laboratory Investigation,* edited by E. J. Farris, and J. Q. Griffith, Jr., 2nd ed., *J. B. Lippincott Co*, 1949, page 431.

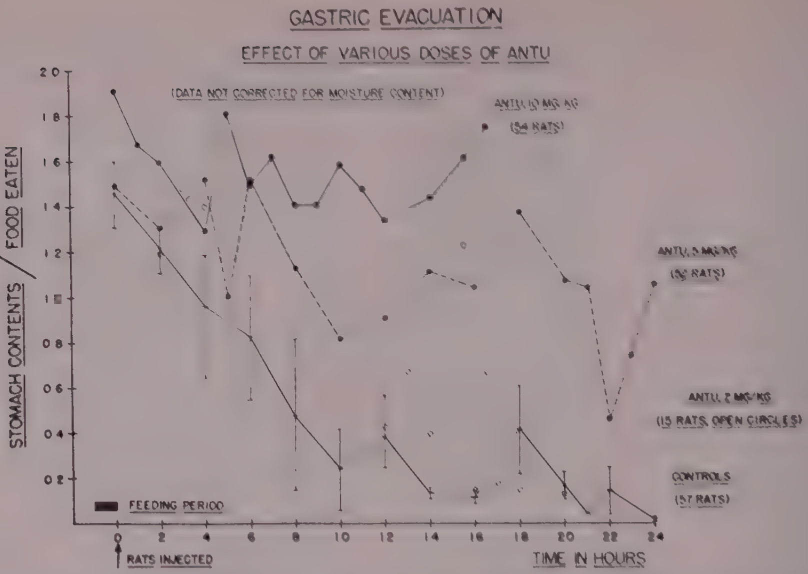


FIG. 1.

Ratios of stomach contents to food eaten, obtained at various times after feeding, when rats were injected with ANTU: 2 mg/kg (open circles, individual values), 5 mg/kg (crossed circles and dotted line, average values), and 10 mg/kg (black circles, average values). All injections intraperitoneal in olive oil. Data *not* corrected for moisture content. Data on control rats (injected with olive oil only) are presented with both average and range.

eaten obtained after 5 mg/kg of ANTU, on the other hand, were found to fall into 2 groups from 5 hours on; one quarter of the rats gave values falling along the control curve, while for the remainder, gastric evacuation was greatly delayed. The irregularity of the 5 mg/kg curve reflects the averaging of disparate values. Rats receiving this dose survived longer than those receiving 10 mg/kg of ANTU, and accordingly the data extend over the full 24-hour period, with an average ratio of 1.06 being obtained at 24 hours. This is comparable to values obtained with control rats at 4 hours after feeding. After 2 mg/kg of ANTU no long-lasting delay in gastric emptying occurred, although it can be seen that the individual values usually fell on the high side of the control curve, but were never as high as ratios obtained after the larger doses of ANTU.

It would seem then that abnormal retention of food in the stomach occurs characteristically after a fatal dose of ANTU. Some of the values obtained at 20 or more hours after poisoning were truly surprising: one poisoned rat ate 12.7 g of food and when sacrificed at 22 hours had 16.4 g in its stomach, as compared to a control that ate 12.2 g and had only 0.2 g remaining at 22 hours. Another poisoned rat ate 29.3 g of food and had a stomach containing 27.6 g 20 hours later.

To determine what fraction of the stomach contents, as measured above, was food and what secretion, an experiment was performed in which dry as well as wet weights of stomach contents were obtained, using 35 additional rats: There were 14 controls, while 11 received 10 mg/kg ANTU, and 10 received 10 mg/kg banthine (a non-fatal dose: 12 additional rats received 10 or 20 mg/kg of banthine and showed no toxic symptoms during the following 48 hours). These injections were again all intraperitoneal, with olive oil as the vehicle. Fig. 2A gives the average values obtained, while the ratios plotted are now for food and for stomach contents after drying.**

** The food itself was found to contain 9.1% moisture, and this fact was taken into account in computing dry weights of food to compare to the dried stomach contents.

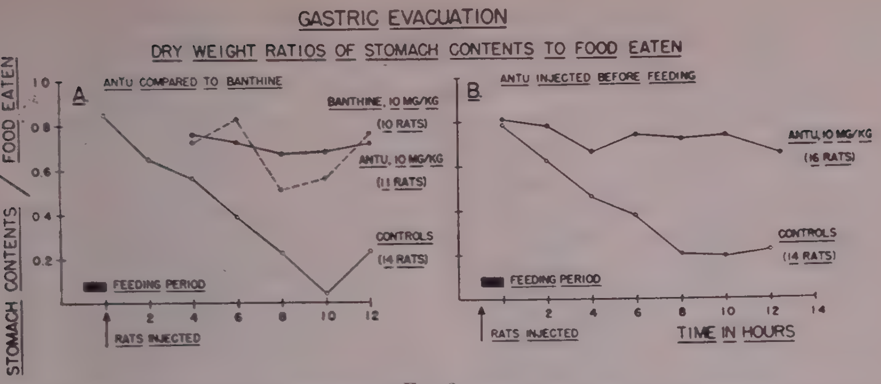


FIG. 2.

Ratios of stomach contents to food eaten, obtained at various times after feeding. Data all corrected for moisture in food and stomach. All injections intraperitoneal in olive oil. A—Comparison of delay produced in control rats, in rats receiving 10 mg/kg of banthine (dotted circles) and in rats receiving 10 mg/kg of ANTU (black circles). B—Effect of ANTU given just before rather than just after feeding.

It will be noted that ANTU and banthine both delayed gastric evacuation, and to an equivalent degree if allowance is made for individual variation.

When the individual dry and wet ratios were compared and then averaged, it was found that the reduction obtained by drying amounted to 54 ± 3% for the controls, 50 ± 4% for the ANTU-treated rats, and 52 ± 5% for those receiving banthine. This variation is sufficiently small to indicate that a rat's stomach usually contains roughly equal amounts of food and secreted fluids, and that division of all the data of Fig. 1 by a factor 2 will correct fairly adequately for moisture content. It is noteworthy that data corrected for moisture content (Fig. 2) give much smoother curves than uncorrected data (Fig. 1).

Fig. 2A also shows that rats sacrificed directly after eating have already discharged about 15% of the ingested food from the stomach. Since ANTU is a relatively insoluble substance which requires several hours after injection before it builds up to toxic levels in the body, it was thought that by injecting ANTU before feeding somewhat higher amounts of food might be retained in the stomach. Accordingly, an experiment was performed in which the rats were injected just before feeding; 14 rats receiving only olive oil served as controls, while 16 received 10 mg/kg of ANTU. The results are shown in Fig. 2B, where the ratio of dried stomach contents to dried food is again plotted against time after feeding ended. Comparison with Fig. 2A shows that no substantially increased retention of food in the stomach occurred, despite the earlier administration of the ANTU.

The experiment using epinephrine (in peanut oil, nearly fatal doses of 3-4 mg/kg injected intraperitoneally) showed that this drug also delayed gastric emptying for the first 12 hours after feeding, giving average "wet" ratios of stomach contents to food eaten ranging from 1.80 to 1.48, but at 16 to 21 hours the effect had worn off and the values obtained (0.17 to 0.09) were equivalent to those of the corresponding controls.

An attempt to repeat these experiments with dogs was unsuccessful, owing to the emetic properties ANTU has even when given intraperitoneally. (The technic was satisfactory for rats because they cannot vomit).

Discussion. The explanation for this action of ANTU in prolonging the sojourn of food in the stomach is as yet unknown. The magnitude of the effect appears from the above results to be related to the acute toxicity, but the relation of this finding to other acute effects of ANTU poisoning, namely the production of pulmonary edema and pleural effusion(4), the disturbance of carbohydrate metabolism as evidenced by a high blood sugar and depleted stores of glycogen in the liver (5), and the rapid drop in body temperature (6), is not clear. It might be mentioned that the data reported above on the moisture content of the rats' stomachs after eating are so nearly identical in rats poisoned with ANTU and in the controls as to indicate that no part of the liquid in the poisoned rats' stomachs was edema fluid. Nor was edema of the stomach wall specifically noted by Latta in his histological studies on ANTU-poisoned rats(4). Sections which might show edema of the pylorus were, however, not included in his material.

It is possible that this delayed gastric evacuation is the result of a direct central action of ANTU, although evidence for a vomiting center in rats is lacking(7). Or it may be mediated reflexly through the autonomic innervation of the stomach, as are the equivalent effects of banthine(8) and epinephrine in this connection.

When administered chronically, ANTU affects the thyroid, causing hyperplasia: it presumably interferes with the production of thyroid hormone just as do the thiouracils and other related anti-thyroid drugs(9). This effect also occurs after a single dose(10); the delayed gastric emptying may therefore be mediated through the thyroid. In this connection it is interesting to consider the work of Northrup and Van Liere(11), who found that sulfapyridine had a statistically significant influence in delaying the emptying of the normal human stomach.

Another possibility is that with the development of pulmonary edema, the rats suffered from anoxia and that this was what delayed gastric evacuation. Van Liere, Cresler and Robinson(12) have found (as later confirmed by Van Liere) (13) that gastric emptying was delayed in dogs subjected to reduced pressures, the more so the less oxygen available. A normal emptying time of 7 to 7.5 hours at atmospheric pressure was increased to more than 24 hours in 2 of their dogs held at somewhat less than half that pressure. This is the only instance a search of the literature has provided of delays in gastric evacuation equal to those reported in this paper.

Summary. Intraperitoneal injection of fatal or near-fatal doses of alpha-naphthyl thiourea (ANTU) markedly lengthened gastric emptying time in rats, as determined by comparing the weights of stomach contents in ANTU-poisoned rats to those of controls. In some rats only minimal evacuation of the stomach had occurred after 24 hours. The chemical vagotomizing agent banthine, in sublethal doses, and epinephrine, in near fatal doses, also delayed gastric evacuation, although the effect of epinephrine wore off before that of ANTU. The explanation for this finding and its relation to other effects produced by ANTU are still unknown.

---

4. Latta, H., *Bull. Johns Hopkins Hosp.*, 1947, v80, 181.

5. DuBois, K. P., Herrmann, R. G., and Erway, W. F., *J. Pharm. and Exp. Therap.*, 1947, v89, 186.

6. Richter, C. P., *Proc. Laurentian Hormone Conf.*, 1948, vII, 255.

7. Hatcher, R. A., *Physiol. Rev.*, 1924, v4, 495.

8. Longino, F. H., Grimson, K. S., Chittum, J. R., and Metcalf, B. H., *Gastroenterology*, 1950, v14, 301.

9. Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, v78, 79.

10. Byerrum, R. U., Cochran, K. W., and DuBois, K. P., PROC. SOC. EXP. BIOL AND MED., 1950, v73, 661.

11. Northrup, D. W., and Van Liere, E. J., *J. Pharm. and Exp. Therap.*, 1940, v70, 297.

12. Van Liere, E. J., Crisler, G., and Robinson, D. H., *Arch. Int. Med.*, 1933, v51, 796.

13. Van Liere, E. J., *Physiol. Revs.*, 1941, v21, 307.

Received March 28, 1951. P.S.E.B.M., 1951, v76.

Localization and Persistence of Toxoplasma in Tissues of Experimentally Infected White Rats.* (18635)

ISAAC RUCHMAN AND JOHN C. FOWLER.

From the Department of Bacteriology, College of Medicine, University of Cincinnati, and the Cincinnati General Hospital.

Jacobs and Jones(1) recently reported the occurrence of parasitemia in various animals inoculated with toxoplasma. In rats the observed parasitemia was sporadic though parasites persisted in the tissues long after the blood was free. In our experiments we wish to report the finding of toxoplasma regularly in the blood stream of experimentally infected rats and of the localization of the parasites in other tissues of the body, especially, their persistence in the brain for as long as two years.

Materials and methods. The R.H. strain of toxoplasma(2) was used throughout. The brains of mice that succumbed after intracerebral inoculation were removed on the fourth day and ground in saline solution to make a 10% suspension. After the larger particles had settled out, the supernatant fluid was used to infect the rats. Intracerebral inoculation into mice of decimal dilutions of this suspension revealed titers of $10^{-5.3}$ to $10^{-5.5}$. Albino rats weighing between 200 and 350 g were inoculated intraperitoneally with one cc of the 10% suspension and they developed inapparent infection. They were sacrificed at various intervals and blood was removed by cardiac puncture. One part of heparin (1:500) was added to 9 parts of blood to inhibit clotting, and 10-fold dilutions of the blood were prepared in saline solution. The animal was then opened and tissues were removed aseptically. Separate instruments were used for each tissue. At first only a portion of each tissue was used but in later experiments the entire organ was removed and made into a 10% suspension either by grinding with mortar and pestle or by homogenizing in a Waring blendor. The presence of toxoplasma was established by subinoculating one cc of the blood or tissue suspensions intraperitoneally into groups of mice and recording the animals that died. Tissue suspensions and dilutions were prepared in saline solution. Frequent checks were made for the presence of toxoplasma by the microscopic examination of Wright stained smears of mouse brains. Surviving mice were tested for immunity by intracerebral inoculation of a 10% suspension of infected mouse brain but all of them died.

Occurrence in the blood stream. After a preliminary experiment had indicated that organisms could be demonstrated in the blood stream within several hours after intraperitoneal inoculation, later experiments were designed to test for the presence of toxoplasma at 4 hours and at daily intervals thereafter (Table I). Organisms were found in the blood at 4, 24, 48 and 72 hours. They were also found in some instances in dilutions of at least 1:10. It was at the fourth and fifth days, however, that the maximal number of organisms was found in the circulating blood. Dilutions of 1:100 and in one instance 1:1000 caused toxoplasmic infection in mice. After the sixth day organisms were present sporadically in the blood stream up to the sixteenth day. The blood was not examined beyond the fourth week.

Occurrence in the viscera. At 4 hours subinoculation of the liver, spleen and lung showed that parasites were present. Toxoplasma were found regularly for nearly a week and more or less regularly for a few more days in liver and spleen 24 hours after inoculation and in lung 48 hours after inoculation. Up to the tenth week they were discovered occasionally in the liver, up to the fourteenth day in the spleen and up to the tenth week in the lungs. The viscera were not examined beyond the twelfth week.

---

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

1. Jacobs, L., and Jones, F. E., *J. Infect. Dis.*, 1950, v87, 78.

2. Sabin, A. B., *J.A.M.A.*, 1941, v116, 801.

TABLE I. Distribution of Toxoplasma in Tissues of Rats Following Intraperitoneal Inoculation. Composite of several tests.

Rat tissue subinoculated into mice	Result of subinoculation of rat tissues into mice at*																												
	Hours					Days																Weeks							
	4	24	48	72	96	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	3	4	5	6	7	8	10	12
Blood																													
Undiluted	6/11	4/10	10/11	3/9	13/13	11/11	6/10	4/11	0/6	2/8	1/8	4/8	1/8	0/8	0/8	0/3	1/5	0/3	0/3	0/3	0/3	0/3	0/3	—	—	—	—	—	—
1:10	0/9	2/9	5/9	3/9	8/9	6/6	2/6	0/6	0/3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1:100	0/9	0/9	1/6	0/6	4/6	5/6	0/6	0/6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1:1000	—	—	—	—	1/6	0/6	0/6	0/6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10% suspensions																													
Liver	3/9	8/9	9/9	8/9	9/9	9/9	9/9	9/9	0/3	2/3	1/3	3/3	1/3	1/3	0/6	—	—	—	—	—	—	0/6	0/6	0/6	3/6	0/6	0/3	1/3	0/3
Spleen	2/9	7/9	9/9	5/9	9/9	8/9	6/9	7/9	2/3	3/3	1/3	3/3	0/3	0/3	1/6	—	—	—	—	—	—	0/6	0/5	0/6	0/6	0/6	0/3	0/3	0/3
Lung	2/9	1/9	6/9	3/9	9/9	9/9	6/9	6/9	3/3	3/3	3/3	3/3	2/3	1/3	5/6	—	—	—	—	—	—	3/6	1/6	0/6	0/6	1/6	0/3	1/3	0/3
Brain	0/9	0/9	2/9	0/9	7/9	7/9	6/9	7/9	3/3	3/3	3/3	3/3	3/3	3/3	3/3	—	—	—	—	—	—	6/6	6/6	6/6	6/6	6/6	3/3	3/3	3/3

* Numerator denotes number of mice that died; denominator denotes number of mice inoculated.

TABLE II. Persistence of Toxoplasma in Brains of Rats.

Interval tested, mo.	Result of subinoculation into mice Brain suspension			
	1:10	1:100	1:1000	1:10000
4	6/6	–	–	–
5	6/6	–	–	–
6	6/6	–	–	–
11	5/5	–	–	–
12	2/2	2/2	1/2	–
18	–	4/4	2/4	0/4
24	4/4	4/4	3/4	–

Occurrence in the brain. For the first few days practically no organisms were detected in the brain (Table I), but on the fourth day and up to a period of 2 years, the longest interval of time studied, the parasites were present with great regularity. In order to obtain a rough idea of their concentration in the brains at the end of one, 1½ and 2 years, 10-fold dilutions of brain were prepared in saline and subinoculated into groups of mice. As seen in Table II organisms were present not only in the 10% suspension but also in dilutions of 1:100 and 1:1000, indicating that although the animals were apparently well they still harbored large numbers of organisms in the brain.

Rat to rat passage by peripheral inoculation. Having decided that organisms were harbored indefinitely in the brains of rats it next became of practical interest to determine if toxoplasma could be maintained by rat to rat passage especially when the interval between passages was long. A rat injected intraperitoneally (Passage I) with infected mouse brain material was sacrificed at the end of one year (Table III). A suspension of its brain was transferred to other rats by the peritoneal route (Passage II). Simultaneously, inoculation of this suspension into mice revealed a titer of $10^{-3.0}$. The rats of passage II were sacrificed at 6 months and 18 months and parasites were demonstrated in the brains. Inoculation of the brain of the animal sacrificed at 6 months was made into 2 rats (Passage III) and into mice which yielded a titer of $10^{-3.0}$. One of the rats of passage III was killed at the end of one year and as soon as it became apparent that parasites were still demonstrable in the brain, the other was also sacrificed. A suspension of its brain was transferred to rats (Passage IV). Again organisms were present in the brain as shown by subinoculation into mice. Passage IV rat was sacrificed at the end of 2 months and titration of its brain in mice showed a titer of $10^{-2.7}$. Thus after 4 consecutive passages in rats which consumed a total of 32 months, the number of organisms present in the rat brain remained undiminished. This indicated that the parasites had not only survived but that they had multiplied. Further rat passages were not undertaken.

Discussion. Although individual variations occur among infected rats nevertheless a general pattern is detectable. Organisms begin to circulate in the blood stream as early as 4 hours after inoculation and continue to do so for about one week, the peak concentration occurring between the fourth and fifth days. After the first week relatively few organisms are found. This period coincides with the first appearance of antibodies in the blood stream. It has been found that experimentally infected white rats develop complement-fixing antibodies towards the end of the first week (3), cytoplasm-modifying antibodies at the end of one week(4) and neutralizing antibodies at the end of 2 weeks(3). As the antibodies appear, the organisms disappear from the blood stream but they may remain longer in some of the tissues of the viscera. Owing to their "protected" environment in the brain toxoplasma may survive in this tissue indefin-

---

3. MacFarlane, J. O., and Ruchman, I., unpublished data.

4. Sabin, A. B., and Feldman, H. A., *Science,* 1948, v108, 660.

TABLE III. Multiplication and Survival of Toxoplasma in Brains of Rats Following Rat to Rat Passage.

Passage No.	Suspension inoculated*	Date		Time interval, mo.	Results with rat brain suspension	
		Inoculated	Sacrificed		Intraperitoneal titer in mice†	Passed to rats
I	10% mouse brain	4- 9-48	3-17-49	11	$10^{-3.0}$	Yes
II	10% rat brain	3-17-49	9-22-49	6	$10^{-3.0}$	Yes
			12- 7-50	18	$10^{-1.5+}$	No
III	,, ,, ,,	9-22-49	9-22-50	12	$10^{-1.5+}$	No
			10- 6-50	12	$10^{-1.5+}$	Yes
IV	,, ,, ,,	10- 6-50	12- 7-50	2	$10^{-2.7}$	No

* 3 cc intraper.

† 1 cc intraper. into groups of mice.

itely. The occurrence of chronic toxoplasmosis in wild rats has already been reported(5). The long residence of organisms in the brain and the known tendency of rodents to devour brain tissue suggests one method of maintaining a reservoir of infection in nature. The fact that toxoplasma can be found in the brains of experimentally infected white rats for periods of at least 2 years becomes of practical importance to investigators working with the organism. For many years it has been the usual procedure to maintain toxoplasma by alternate storage in the refrigerator for 2 weeks and then passage into mice especially when uniformly fatal strains are used. In this laboratory it is now more convenient to inoculate rats. Whenever the organisms are required, a rat is sacrificed and its brain passed to mice. After one or two passages the organism is ready for use. Additional rats may then be inoculated intraperitoneally with this material. Thus the white rat may be used to demonstrate a parasitemia or an antibody response, which can be determined by several serological methods. It also serves as an excellent means of maintaining the organisms conveniently and for long periods of time, and finally it can be used to study the carrier state.

Summary. Intraperitoneal inoculation of toxoplasma into rats yielded the following data. Organisms were shown to be present in the blood 4 hours after inoculation and daily thereafter for the next week. The maximal concentration was reached between the fourth and fifth days. Organisms were found sporadically during the next few weeks. Parasites were present in small numbers in the liver, spleen and lungs at 4 hours but were more regularly found after that for 7 days in the liver, 9 days in the spleen and 11 days in the lungs. They were less regularly found after these periods of time for 10 weeks in the liver and lungs and for 2 weeks in the spleen. Toxoplasma appeared in the brain at 4 days and were uniformly present thereafter for a period of 2 years, which was the longest interval of time that the tissue was examined. Intraperitoneal inoculation of rats is a convenient method to maintain the parasites indefinitely.

Received March 30, 1951. P.S.E.B.M., 1951, v76.

---

5. Perrin, T. L., Brigham, G. D., and Pickens, E. G., *J. Infect. Dis.*, 1943, v72, 91.

Interrelation between Potassium Metabolism and Digitalis Toxicity in Heart Failure.[*] (18636)

Bernard Lown,[†] Herbert Salzberg,[‡] Charles D. Enselberg,
and Raymond E. Weston.

From the Medical Division, Montefiore Hospital, New York City

A relationship between potassium metabolism and the cardiac effects of digitalis has long been recognized. In isolated heart muscle preparations, digitalis action can either be enhanced or inhibited by appropriate changes of potassium concentration(1). A number of investigators have reported that orally administered potassium temporarily abolishes electrocardiographic manifestations of digitalis intoxication in patients(2,3). Whether these represent transient pharmacologic effects or physiologic restitution of intracellular potassium depletion has not been established. Reevaluation of certain clinical observations in terms of present understanding of electrolyte distribution suggests that diminution of myocardial potassium concentration alters the response of the heart to digitalis. Increased sensitivity to digitalis to the point of toxicity is encountered after mercurial diuresis, acute myocardial infarction, pulmonary embolism, acute infections, etc. In each of these circumstances, negative potassium balance has been observed either as a result of a mercurial induced loss(4) or as a part of the bodily response to acute stress(5). Similarly, the normal, in contrast to the failing, heart is resistant to the development of signs of digitalis intoxication. Recent work(6) confirms the older observation(7) that the failing heart is depleted of potassium to an extent which is dependent upon the degree of myocardial insufficiency.

The present study is an attempt to determine whether alterations in digitalis sensitivity can be demonstrated following changes in potassium metabolism encountered clinically.

Material and methods. The subjects were 10 patients in various stages of congestive heart failure who were maintained on a low sodium (20-25 milliequivalents) diet. During the control period, in 6 of the patients, the threshold of mild toxicity to a rapidly acting digitalis preparation, ouabain or acetyl strophanthidin[§] was determined. The electrocardiographic criteria of digitalis toxicity were ventricular extrasystoles, impairment of auriculo-ventricular conduction, or alterations in the pacemaker. The short acting glycosides facilitate early electrocardiographic recognition of digitalis toxicity and permit repetition of studies at more frequent intervals. When acetyl strophanthidin was used, a test dose of 0.6 mg was first given. Then, after lapses of at least 48 hours, a second dose of 1.2 mg was injected, and if no toxic signs appeared, a final dose of 1.8 mg was given. Electrocardiograms were taken continuously

---

[*] This study was supported in part by grants from the National Heart Institute, U. S. Public Health Service, and the Martha M. Hall Foundation.

[†] Present address Peter Bent Brigham Hospital, Boston.

[‡] Present address Bronx Veterans Administration Hospital, Bronx, N. Y.

1. Friedman, M. and Bine, R., Jr., *Am. J. Med. Sc.*, 1947, v214, 633.

2. Sampson, J. J. and Anderson, M. E., *J.A.M.A.*, 1932, v99, 2257.

3. Enselberg, C. D., Simmons, G. H., Mintz, A. A., *Am. Heart J.*, 1950, v39, 713.

4. Blumgart, H. L., Gilligan, D. R., Levy, R. D., Brown, M. G., and Volk, M. C., *Arch. Int. Med.*, 1934, v54, 40.

5. Sayers, G., *Physiol. Rev.*, 1950, v30, 241.

6. Alexander, L. C., Boyle, J. A., Iseri, L. T., McCaughey, R. S., and Myers, G. B., *Proc. Central Soc. for Clin. Invest.*, 1950, v23, 2.

7. Harrison, T. R., Pilcher, C. and Ewing, G., *J. Clin. Invest.*, 1930, v8, 325.

[§] Acetyl strophanthidin is a synthetic ester of the aglycone strophanthidine. Its cardiac effect in man is evident within 1-5 minutes, reaches a peak at about 16 minutes and generally is dissipated in 60 minutes. The usual intravenous digitalizing dose is 1.2 to 1.8 mg.(8)

8. Enselberg, C. D., Altchek, R. N., and Hellman, E., *Am. Heart J.*, 1950, v6, 919.

TABLE I. Summary of Results.

Patient	Agency inducing K change	Serum K, m.eq./l	Gross K balance	Digitalis dosage, mg	Digitalis toxicity
H.D.	a. 0	5.2	Unchanged	1.2A*	0
	b. 0	5.7	”	1.2	0
	c. Amm. chloride and thiomerin	5.0	—83 m.eq.	1.2	Vomiting. Multifocal VPBs. Runs of ventricular tachycardia
H.S.	a. 0	5.6	Unchanged	1.8A	0
	b. 0	4.8	”	1.8	0
	c. Amm. chloride and thiomerin	4.7	”	1.8	0
S.S.	a. 0	4.8	”	1.8A	0
	b. 0		”	1.8	0
	c. Amm. chloride and thiomerin	4.9	”	1.8	0
A.R.	a. Amm. chloride and thiomerin	5.1	—100 m.eq.	0.7O†	Vomiting. Changing pacemaker. Complete A-V block. Rare VPB
	b. Amm. chloride and thiomerin	3.6	— 70 ”	0.7	”
	c. KCL 100 m.eq./day for 7 days	5.2	+210 ”	1.3	”
A.S.	a. Thiomerin (5 observations)	4.8	—	0.25-0.5D‡	Vomiting. Bigeminy
	b. KCL 67 m.eq./day at time of diuretic (3 observations)	4.4	—	0.25-0.5	0
G.R.	a. 0	5.75	Unchanged	1.2A	Nausea and tingling
	b. DCA—20 mg/day for 5 days	5.35	—85 m.eq.	1.2	Nausea, tingling and bigeminy
L.C.	a. 0	4.9	Unchanged	1.8A	0
	b. 0	—	”	1.8	0
	c. DCA—25 mg/day for 10 days	4.8	”	1.8	Nausea, tingling; incomplete A-V block. Occasional VPB
	d. DCA and thiomerin	—	”	1.8	”
W.E.	a. Malnutrition and gastrointestinal loss	5.1	Probable depletion	1.2A	Ventricular flutter-fibrillation
	b. ”		”	0.6	Frequent VPBs in pairs and triplets
A.F.	a. Malnutrition, dehydration & gastrointestinal loss	2.3	”	0.6A	Bigeminy, A-V block, ventricular tachycardia and fibrillation (fatal outcome)
M.F.	a. Glucose and insulin§	4.1 3.0	—	0	Nausea, dizziness, faintness, angina pectoris, multifocal VPBs with pairs and triplets
	b. Glucose and insulin‖	5.0 3.9	—	0	0

* A = Acetyl strophanthidin.
† O = Ouabain.
‡ D = Digoxin.
§ Glucose and insulin given 2 days after loss of digitalis toxicity, 9 days without digitalis.
‖ No digitalis for 6 wk.

for fifteen minutes after the injection, and thereafter at 5 minute intervals for 90 minutes or until disappearance of digitalis effect.

Ouabain was administered as follows: after two 0.25 mg doses, given at a 30 minute interval, 0.1 mg was injected every 30 minutes until signs of mild toxicity were noted. Before, and 5 minutes after each injection of ouabain, 2 minute electrocardiographic records were obtained. After the final dose, which produced evidence of toxicity, similar frequent electrocardiographic records were taken for at least 2 hours. A similar technic with the same glycoside was used to test digitalis sensitivity after the induction of alterations in body potassium by either of the following procedures: in four patients (H.D., A.R., H.S., S.S.) by the administration of the mercurial diuretic Thiomerin, after 3 days of pretreatment with ammonium chloride, which is known to promote increased urinary potassium excretion(9), and in 2 (G.R. and L.C.) by the administration of desoxycorticosterone acetate (DCA).¶ In 2 other patients (W.E. and A.F.) who had been depleted of potassium by vomiting, diarrhea and malnutrition, digitalis sensitivity was tested with subtoxic doses of acetyl strophanthidin. Other experimental details are discussed below.

Blood electrolyte levels and 24 hour urine excretions of creatinine, sodium, chloride and potassium were determined. Chemical analyses were performed by the following methods: chlorides by Van Slyke's modification of Sendroy's iodometric technic(10), sodium and potassium on a lithium internal standard flame photometer; and creatinine by Peter's modification of the Folin-Wu procedure(11).

Results. In each case with significant shifts of potassium changes in digitalis sensitivity were observed (Table I). The gross K balance indicated in Table I refers to changes in urinary potassium excretion, which was relatively constant before the experimental procedures employed to remove or replace body potassium.

In patient H.D. an 83 milliequivalent loss of potassium after ammonium chloride and Thiomerin, without any significant changes in the serum potassium concentration, was associated with markedly increased sensitivity to acetyl strophanthidin. In contrast, the 2 patients (H.S. and S.S.) in whom these diuretics induced no increase in urinary potassium excretion, failed to develop any signs of digitalis overdosage.

In patient A.R. after diuretics, 0.7 mg of ouabain produced toxicity. However, after daily oral administration of 100 milliequivalents of potassium citrate for a week, 1.3 mg of ouabain were required to achieve the same effect. Moreover, there was not only an increase in the threshold for digitalis toxicity, but also a marked reduction in the duration of toxic manifestation. Over a 2 month period patient A.S., while on a constant maintenance dose of digoxin, consistently developed severe toxicity the day following diuretic therapy. Oral administration of 67 m.eq. of potassium citrate on days when mercurials were given, prevented all signs of her usual toxicity 24 hours later.

Increased sensitivity to digitalis was observed in patients G.R. and L.C. after the daily administration of 20-25 mg of DCA for four and ten days, respectively. In patient G.R., who was on a complete metabolic balance study, an 85 m.eq. potassium loss was sustained during the period of DCA administration. In patient L.C. there were no demonstrable changes in potassium excretion.

There was no consistent lowering of serum K levels in any of these patients in whom potassium loss was induced and digitalis sensitivity was enhanced. One (A.F.) of the 2 patients with malnutrition and gastrointestinal potassium loss was hypokalemic, whereas the other (W.E.) had a normal serum potassium concentration. Nevertheless, both exhibited profound digitalis sensitivity. In patient M.F., bigeminy due to an overdosage of digitalis could be abolished by oral potassium and reinduced by mercurial diuresis. Nine days after discontinuing digitalis, when diuretics no longer produced ventricular extrasystoles, 50 g of glucose and 25 units of insulin were injected intravenously over a 15 minute period. The resulting decrease in serum potassium from 4.1 m.eq./l to 3.0

---

9. Gamble, J. L., *Chemical Anatomy, Physiology and Pathology of Extracellular Fluid,* Harvard University Press, Cambridge, Mass., 1950.

¶ Supplied by Schering Corp.

10. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.,* 1947, v167, 107.

11. Peters, J. H., *J. Biol. Chem.,* 1942, v146, 179.

m.eq./l was associated with the development of severe digitalis toxicity, which was abolished by a single oral dose of 130 m.eq. of potassium citrate. She received no more digitalis and 6 weeks later was fibrillating with a ventricular rate of 150. At this time, repeating the same intravenous dose of glucose and insulin induced no signs of toxicity.

Discussion. This study suggests that depletion of potassium increases the sensitivity of the heart to digitalis. The absence of signs of digitalis overdosage when mercurials did not produce an increase in potassium excretion demonstrates that the mercurial itself is not the responsible agent in those instances in which toxicity does ensue. Ordinarily, the reduction of ventricular extrasystoles after oral potassium begins within 30 minutes, is maximal at 2 hours, and subsides within 4 to 8 hours(3). This transient effect of K in the usual case with more persistent signs of digitalis overdosage may represent the continuing operation of the factors which initially produced the underlying intracellular potassium depletion. If potassium intake is maintained at a high level, there may be no recurrence of ventricular extrasystoles. In patient A.S., however, signs of digitalis intoxication occurred only after mercurial induced potassium loss. It is noteworthy that when the latter was corrected by orally administering potassium on the day of diuresis the digitalis induced premature beats usually occurring 24 hours later, did not develop.

From the present data, it would appear that the increased myocardial sensitivity to digitalis observed after potassium losses probably results from alterations in intracellular potassium distribution. Changes in extracellular potassium concentration probably are not of primary importance, since there were no significant changes in serum K levels in all but two of the patients. The decreased tolerance to digitalis observed after DCA was not accompanied by a demonstrable increase in potassium excretion in one of two patients. Unless there is a synergism between this steroid and digitalis(12), the altered digitalis sensitivity in this case may be a reflection of a shift in myocardial potassium(13). In relation to total intracellular potassium, such shifts of cardiac electrolyte, leading to the altered response to digitalis, may be too small to be measured by available technics.

A similar shift in myocardial potassium distribution may be involved in the electrocardiographic signs of digitalis intoxication observed after glucose and insulin when patient M.F. was still digitalized. The marked drop in serum potassium may have promoted a decrease in that fraction of myocardial potassium which is involved in the production of extrasystoles. In patients who had never received any digitalis, analogous electrocardiographic changes have been observed without any previous digitalis therapy, after shifts of potassium induced by glucose injection in patients with hypokalemia(14) or familial periodic paralysis(15), or by prolonged DCA administration(16).

In all previous studies, including our own, the influence of possible simultaneous changes in distribution of water and electrolytes other than potassium cannot be ruled out. However, in preliminary experiments performed by one of us (B.L.) with Merrill in Boston it has been possible to control these variables. With the artificial kidney, by the selective removal of potassium, digitalis toxicity has been induced in digitalized patients, and then abolished by the restoration of the removed potassium. Further studies are in progress.

Summary and conclusion. 1. In a series of patients in congestive failure, potassium depletion, either occurring spontaneously with malnutrition and gastrointestinal disorders or induced by mercurial diuresis after pre-treatment with ammonium chloride, was found to be associated with increased sensitivity to digitalis. 2. In 2 patients a similar decreased threshold of digitalis toxicity was observed after daily doses of 20 mg of desoxycorticos-

---

12. Zwemer, R. and Lowenstein, B. E., *Science*, 1940, v91, 76.

13. Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, 1942, v21, 601.

14. Gass, H., Cherkasky, M. and Savitsky, N., *Medicine*, 1946, v27, 105.

15. Weston, R. E. and Mark, H., Unpublished Observations.

terone acetate for 4 to 10 days. 3. In one digitalized patient, digitalis toxicity was precipitated by intravenous injection of insulin and glucose, which was followed by the development of hypokalemia. 4. It is suggested that this increased myocardial sensitivity to digitalis probably results from alterations in intracellular potassium distribution since no significant changes in serum (or extracellular) potassium were observed in 8 of 10 patients.

The authors gratefully acknowledge the advice and interest of Dr. Louis Leiter, and the technical assistance of Mr. George Ross and Mrs. Lila Wolfman, chemists of the Medical Division Laboratories at Montefiore Hospital.

Received March 27, 1951. P.S.E.B.M., 1951, v76.

Hair Loss from Squalene.* (18637)

PETER FLESCH. (Introduced by P. Gyorgy)

From the Department of Dermatology and Syphilology, School of Medicine, University of Pennsylvania, Philadelphia.

In previous reports the reversible local depilatory action of the intermediary polymers (cyclic dimers) of chloroprene was described (1,2). These compounds, when applied to the skin of laboratory animals, caused localized temporary cessation of hair growth with marked thickening of the epidermis and anomalous keratinization(3). *In vitro* the dimers inactivated the free sulfhydryl groups of glutathione and of tissue homogenates in amounts which corresponded well with the *in vivo* depilatory concentrations. We have attributed the sulfhydryl inactivating action of the dimers to the unsaturated bonds in the molecule which alkylate the –SH group according to the equation: $-CH=CH- + RSH \longrightarrow RS-\overset{|}{C}H-CH_2-$. The theory was advanced that naturally occurring unsaturated compounds may owe their effect upon keratinization to a similar chemical reaction(4). Such a compound is vit. A, a polymer of isoprene; isoprene differs from chloroprene in having a CH_3 group in place of the Cl atom of chloroprene. We have confirmed previous findings that vit. A, in addition to causing generalized hair loss in animals(5-7) and human beings(8-10), has a distinct local depilatory effect(4,11). However, vit. A had no demonstrable effect on free sulfhydryl compounds *in vitro* and it is possible that its depilatory action is due to a metabolic breakdown product.

The present paper deals with the localized reversible depilatory effect of another naturally occurring isoprene polymer, squalene. Squalene, an unsaturated hydrocarbon, is a normal component of human sebum(12) and therefore it may have a direct effect on normal, as well as pathologic, hair formation in man.

---

* This work was done under a Damon Runyon Senior Clinical Fellowship of the American Cancer Society.

1. Ritter, W. L., and Carter, A. S., *J. Ind. Hyg.*, 1948, v30, 192.

2. Flesch, P., and Goldstone, S. B., *Science*, 1951, v113, 126.

3. Flesch, P., Kligman, A. M. and Baldridge, G. D., *J. Invest. Derm.*, 1951, v16, 81.

4. Flesch, P., and Hunt, M., To be published.

5. Strauss, K., *Beitr. Path. Anat.*, 1934-35, v94. 345.

6. Weslaw, W., Wronski, B., Wroblewski, A., and Wroblewski, B., *Klin. Wchschr.*, 1938, v17, 777.

7. Studer, A., and Frey, J. R., *Schweiz. Med. Wchschr.*, 1949, v79, 382.

8. Josephs, H. W., *Am. J. Dis. Child.*, 1944, v67, 33.

9. Toomey, J. A., and Morissette, R. A., *Am. J. Dis. Child.*, 1947, v73, 473.

10. Rothman, P. E., and Leon, E. E., *Radiology*, 1948, v51, 368.

11. Weslaw, W., Wronski, B., Wroblewski, A., and Wroblewski, B., *Klin. Wchschr.*, 1948, v17, 879.

12. McKenna, R. M. B., Wheatley, V. R. and Wormall, A., *J. Invest. Derm.*, 1950, v15, 33.

Experimental. Squalene† was applied without rubbing to the skin of 10 rabbits, 4 guinea pigs (1 ml each) and 16 mice (0.2 ml each). The area between the ears and on top of the head was chosen, as being inaccessible to scratching and licking. In preliminary experiments squalene was mixed with 1% ascaridole (final concentration), since it has been shown that the addition of –SH groups to the unsaturated double bond is catalyzed by organic peroxides(13). In subsequent experiments pure squalene was used with the same results. Sulfhydryl determinations *in vitro* were made with buffered (pH 7.4) aqueous solutions of glutathione‡ (70 μg in 0.2 ml) and in 10% aqueous homogenates of mouse liver and of heat separated human epidermis(14) by using Bennett's reagent(15) and Anson's method(16). The sulfhydryl compounds were incubated with 0.006-0.1 ml squalene at room temperature for 15 minutes with frequent shaking. Measurement of the activity of a sulfhydryl enzyme, succinic dehydrogenase, was carried out with the method of Kun and Abood(17) after incubating 1 ml of 10% aqueous mouse liver homogenate with 0.2-1.0 ml squalene at room temperature for 15 minutes.

Results. After a single application of squalene the hair of all rabbits treated began to fall out in 1 week; complete baldness resulted in 10-12 days. Three out of 4 guinea pigs lost their hair after 10 days. The hair loss was limited to the area of application (Fig. 1). The hair came out by the root in bundles consisting of 3 to 6 hairs, embedded in small white scales. The underlying skin was smooth and finely scaling. In contrast to the depilatory effect of the chloroprene dimers and of the allyl esters(4), depilation by squalene was not accompanied by inflammation and crusting. The first signs of hair regeneration were visible at the beginning of the third week and within a few weeks the fur resumed its normal appearance in all animals. No depilation could be elicited in mice in spite of repeated applications. None of the animals displayed any toxic symptoms.

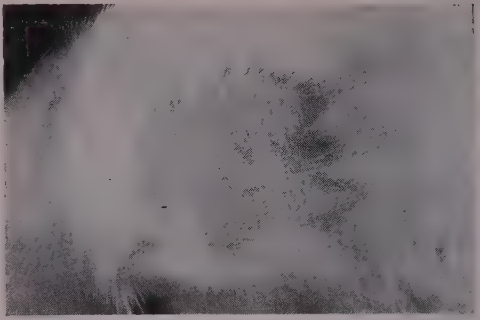


FIG. 1.
Local hair loss in rabbit 14 days after single topical application of 1 ml squalene.

Hairs immersed in squalene for several weeks showed no macroscopic or microscopic signs of disintegration. The action of squalene is therefore similar to that of the other unsaturated depilatory compounds which have no keratolytic effect.

Biopsy specimens of rabbit skin taken 12 days after application of squalene revealed a hyperplasia of the cutaneous epithelium. This was especially pronounced in the more active cells, namely the bulbs of the hair follicles; here it was of adenomatoid proportions. The hair shafts had either disappeared completely or only keratinous shreds remained in the upper parts of the follicles. Many of the deeper follicular cells developed eosinophilic (keratohyalin?) granules. There was no inflammatory reaction of significance, at the most only scattered inflammatory cells under the epidermis without edema.

Avulsed hairs, examined *in toto* under the microscope, showed the features of normal exfoliative hairs with the roots embedded in keratotic (not exudative) scales.§

In vitro, squalene inactivated the free

---

† Obtained through courtesy of Dr. M. Hunt, DuPont and Co.

13. Kharasch, M. S., Reid, A. T., and Mayo, F. R., *Chem. and Ind.*, 1938, v16, 752.

‡ Schwartz Laboratories, New York City.

14. Baumberger, J. P., Suntzeff, V., and Cowdry, E. W., *J. Nat. Cancer Inst.*, 1942, v2, 413.

15. Flesch, P., and Kun, E., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 249.

16. Anson, M. L., *J. Gen. Physiol.*, 1941, v24, 399.

17. Kun, E., and Abood, L. G., *Science*, 1949, v109, 144.

§ The author is greatly indebted to Dr. Fred D. Weidman for the microscopic studies.

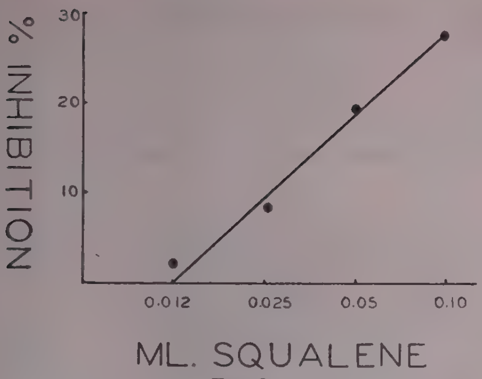


FIG. 2.

Inactivation of free sulfhydryl groups of glutathione by squalene.

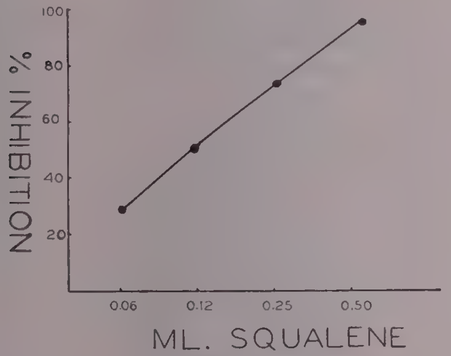


FIG. 3.

Inhibition of succinic dehydrogenase activity of 100 mg of mouse liver homogenate by squalene.

sulfhydryl groups of glutathione, human epidermis and mouse liver homogenates. The percentage inactivation gave a linear relationship when plotted against milliliters of squalene on semilogarithmic paper (Fig. 2). Inactivation of succinic dehydrogenase followed a similar curve. (Fig. 3).

Discussion. The finding that squalene causes local depilation in rabbits and inactivates sulfhydryl compounds *in vitro,* further supports the theory that hair growth can be inhibited by unsaturated compounds which alkylate the free sulfhydryl groups. This reaction between squalene and sulfhydryl compounds apparently occurs under milder conditions than has been described previously (18). In view of the non-irritating nature of squalene, the hair loss cannot be ascribed to the secondary effect of inflammation. At present no explanation can be offered for the lack of depilatory activity in mice. Species differences have been found previously with the allyl esters which depilate mice, but have no effect on rabbits(19).

The question whether or not squalene or a related unsaturated compound in sebum may cause disturbances in human hair growth, cannot be answered at present. Such a possibility is supported by our previous observation that application of the chloroprene dimers in mice for several months may result in irreversible thinning of the hair(19). Theories concerning the relationship of common male baldness with sebaceous dysfunction have been advanced previously(20,21). In view of the present findings, these theories should be reevaluated by quantitative measurements of unsaturated compounds in the sebum of normal children and adults and of persons with incipient baldness. Studies of this nature are in progress. Clinical trials with squalene in the treatment of fungous infections of the scalp have been started.

Summary. Squalene, an isoprene polymer occurring in normal human sebum, caused complete reversible local depilation in rabbits and guinea pigs after a single topical application. No hair loss was observed in mice. *In vitro,* squalene inactivated the free sulfhydryl groups of glutathione, human epidermis and mouse liver and inhibited succinic dehydrogenase activity of mouse liver. The depilatory and sulfhydryl inactivating effects of squalene are believed to be due to alkylation of the sulfhydryl group by the unsaturated double bonds in the molecule. The possibility is raised that squalene or a related unsaturated compound in sebum may influence human hair growth.

---

18. Cunneen, J. I., *J. Chem. Soc.,* 1947, 36.

19. Flesch, P., Unpublished observations.

20. Sabouraud, R., *Maladies du cuir chevelu. I. Les maladies séborrhéiques.* Paris, Masson & Co., 1902.

21. Rothman S., Personal communication.

Received March 27, 1951. P.S.E.B.M., 1951, v76.

Effect of Riboflavin and Pteroylglutamic Acid on Growth and White Cell Production of Rats.* (18638)

Barbara Kelley, Martha Northrup, and Patricia Davis Hurley.
(Introduced by P. L. Day)

From the Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock, Ark.

Previous work from this laboratory has indicated that the growth depression brought about by feeding a high (10%) level of glycine to rats can be partially overcome by the addition of pteroylglutamic acid (PGA)(1). It has also been reported by Martel and Gingras(2) that the feeding of a 10% level of glycine to rats resulted in a mild leucopenia which responded to PGA. The present experiments were undertaken in an effort to determine if riboflavin or choline could substitute for PGA in these functions. Since glycine oxidase is a flavin-containing enzyme riboflavin could possibly increase the rate of destruction of the glycine. It has been shown that one or more of the methyl groups of choline may be converted to a "one-carbon fragment" and condensed with glycine to form serine(3).

Experimental. Nine groups of male weanling Sprague-Dawley rats were fed diets of the composition given in Table I. Each animal was given 6 g of food per day for the first 16 days; 7 g per day from the 16th-32nd experimental day; and 8 g per day from the 32nd-56th day. The supplements of riboflavin and PGA were added daily to the food. On the restricted food intake employed in these experiments the animals of all groups consumed most of the food and supplements given them. Complete blood counts were made on the 32nd day of the experiment. The differences in weight gain and in white blood cells were statistically treated by the analysis of variance method(4).

Results and discussion. The average weight gains and the weight gains per g of food eaten for the various groups are given in Table II. It is evident from these data that neither a high level of riboflavin (Group V) nor a high level of choline (Group VII) had any effect in preventing the growth suppression brought about by the toxic level of glycine. The combination of choline and riboflavin (Group VIII) was also ineffective. PGA was found to partially but not completely overcome this growth suppression when given along with the riboflavin. The response with the combination of riboflavin and PGA, however, was no better than that reported by Dinning *et al.* (1) using PGA alone on high glycine diets. It would thus seem that riboflavin cannot replace PGA nor does it act synergistically with it in preventing the growth suppression brought about by the inclusion of 10% glycine in the diet.

The hematological data are presented in Table II. A mild leucopenia developed on the 10% glycine diet which is in accord with the results of other investigators(1,2). The addition of riboflavin to the high glycine and high glycine plus choline diets brought about a reduction in white cells as compared to the unsupplemented controls. This reduction was not significant in the animals receiving a high level of both glycine and choline. In all cases where additional riboflavin and PGA were supplied in the diets (Groups III, VI, and IX) there was a statistically significant increase in the white cell count over that of the basal group and also over that of their

---

* Research paper No. 956, Journal Series, University of Arkansas. This investigation was supported in part by a research grant from the National Institutes of Health, United States Public Health Service.

1. Dinning, J. S., Keith, C. K., Day, P. L., and Totter, J. R., Proc. Soc. Exp. Biol. and Med., 1949, v72, 262.

2. Martel, F., Page, E., and Gingras, R., *Rev. Canadienne de Biologie,* 1947, v6, 802.

3. Sakami, W., *J. Biol. Chem.,* 1949, v179, 495.

4. Snedecor, George W., *Statistical Methods Applied to Experiments in Agriculture and Biology,* The Iowa State College Press, 4th ed., 1946.

TABLE I. Composition of Diets.

Components	I	II	III	IV	V	VI	VII	VIII	IX
					%				
Casein	18	18	18	18	18	18	18	18	18
Sucrose	68	68	68	58	58	58	56	56	56
Crisco	10	10	10	10	10	10	10	10	10
Cod liver oil	2	2	2	2	2	2	2	2	2
Salt mix	2	2	2	2	2	2	2	2	2
Glycine	0	0	0	10	10	10	10	10	10
Choline	0	0	0	0	0	0	2	2	2
					Supplements				
Riboflavin, γ/day	0	100	100	0	100	100	0	100	100
Folic acid, γ/day	0	0	100	0	0	100	0	0	100

The following vitamins were added per 100 g to all diets:

	mg		mg
Thiamine	.5	Choline chloride	10
Riboflavin	.5	Pyridoxine hydrochloride	.5
Nicotinic acid	2.	Biotin	.005
i-Inositol	10.	Menadione	.025
Calcium pantothenate	1		

TABLE II. Weight Gain and Hematological Data of Rats Receiving Riboflavin and Folic Acid on Various Purified Diets.

Group*	Diet	Avg wt gain 56 days, g	Wt gain per g food eaten, g	R.B.C., 10^6/mm^3	W.B.C.,† 10^3/mm^3	Lymphocytes, 10^3/mm^3
I	Basal	156	.354	8.30	16.58	13.81
II	Basal + riboflavin	153	.358	8.18	17.28	14.06
III	Basal + riboflavin + PGA	152	.356	7.97	20.82	17.89
IV	High glycine	85	.235	7.92	14.05	11.08
V	" " + riboflavin	78	.211	7.92	13.60	9.50
VI	" " + " + PGA	132	.322	8.77	19.55	15.43
VII	High glycine + choline	85	.252	7.85	19.37	15.89
VIII	High glycine + choline + riboflavin	94	.258	8.79	14.60	11.42
IX	High glycine + choline + riboflavin + PGA	127	.319	9.30	25.44	20.94

* Six or 7 animals in each group.

† A difference of 2.37 × 10^3 white blood cells per mm^3 is statistically significant (P = 0.01).

unsupplemented controls. In the present experiments the increase in white cell production brought about by riboflavin and PGA was much greater when there was a high level of dietary choline (Group II). This would indicate that choline or some product of choline metabolism influences such cell production. That PGA alone will not bring about this increase with high levels of glycine, choline or a combination of these substances is indicated by the results shown in Table III. Recent work has indicated that "methyl" groups are necessary for the formation of white blood cells and that these methyl groups can be supplied by betaine when there is sufficient vit. B_{12} present(5). In the present experiments casein was used as the protein source which would supply sufficient vit. B_{12} to meet the requirements of the rat. A relationship of PGA to flavin-containing enzymes (6) and to choline oxidase(7) has been postulated. Results such as those obtained in the present experiments might be explained on

---

5. Dinning, J. S., Payne, L. D., and Day, P. L., *Arch. Biochem.*, 1950, v27, 467.

6. Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, 1948, v176, 1095.

7. Dinning, J. S., Keith, C. K., and Day, P. L., *J. Biol. Chem.*, 1951, v189, 515.

TABLE III. Hematological Data on Rats Receiving High Levels of Glycine and Choline With and Without Added PGA.

Diet Experiment I	No. of animals	R.B.C. 20th day $10^6/mm^3$	W.B.C. $10^3/mm^3$
Basal	4	7.63	17.8
Basal + 500 γ PGA/100 g	7	7.30	18.7
Basal + 10% glycine	5	7.43	18.0
Basal + 10% glycine + 500 γ PGA/100 g	5	7.10	16.6
Basal + 10% glycine + 2% choline	5	9.23	17.1
Basal + 10% glycine + 2% choline + 500 γ PGA/100 g	5	9.78	17.9
Experiment II		32nd day	
Basal	8	8.31	18.8
Basal + 6% choline	8	8.49	18.9
Basal + 6% choline + 500 γ PGA/100 g	8	8.84	17.8

the basis of an altered rate of conversion of choline to betaine.

The red cell counts, hemoglobin and hematocrits of the various groups showed no significant differences.

Summary. Weanling albino rats were fed a standard purified diet, a similar diet with 10% glycine replacing an equal amount of sucrose, and a diet with 10% glycine and 2% choline replacing an equal amount of sucrose. Other groups received these diets supplemented with 100 γ of riboflavin per day or 100 γ of riboflavin and 100 γ of PGA per day. The growth of the rats receiving the high glycine and high glycine plus high choline diets was greatly reduced as compared to the basal group. Supplementation of these diets with riboflavin brought about no effect on the growth; the combination of riboflavin and PGA overcame the growth depression to a considerable extent and also brought about a marked increase in gain per unit of food consumed. Supplementing the various diets with both riboflavin and PGA brought about a leucocytosis which was very marked when the diet contained a high level of both glycine and choline. The data would suggest that there is an interrelationship between riboflavin and PGA in white cell production.

Received March 27, 1951. P.S.E.B.M., 1951, v76.

Further Observations on the Microbiological Assay for Vitamin B_{12} Using *Lactobacillus leichmannii.* (18639)

HARRY P. BROQUIST, E. L. R. STOKSTAD, AND T. H. JUKES.

From the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Conflicting reports have appeared regarding the comparative potencies of vit. B_{12} and B_{12b} in microbiological assays with *Lactobacillus leichmannii* and *L. lactis* Dorner. The two compounds were found to have approximately equal activity for *L. leichmannii* by Pierce *et al.*(1,2). However, in subsequent studies in our laboratories, the potency of vit. B_{12b} often appeared to be only 0.7 to 0.8 that of vit. B_{12} in the *L. leichmannii* assay. In contrast, Jackson and co-workers(3) found that vit. B_{12b} was 1.4 to 1.5 times as potent as vit. B_{12} when the vitamins were added

---

1. Pierce, J. V., Page, A. C., Jr., Stokstad, E. L. R., and Jukes, T. H., *J. Am. Chem. Soc.*, 1950, v72, 2615.

2. Fricke, H. H., Lanius, B., DeRose, A. F., Lapidus, M., and Frost, D. V., *Fed. Proc.*, 1950, v9, 173.

3. Jackson, W. G., Whitfield, G. B., DeVries, W. H., Nelson, H. A., and Evans, J. S., *J. Am. Chem. Soc.*, 1951, v73, 337.

aseptically to the culture media. Vit. B_{12b} had about 60% of the activity of vit. B_{12} for *L. lactis* Dorner when autoclaved with the medium but the two substances showed equal activity when added aseptically. Kaczka *et al.*(4) reported the following relative values for vit. B_{12b} with different assay organisms assigning vit. B_{12} a potency of 1.0: with *L. lactis* 0.62 (titrimetric), 1.0 (cup), with *L. leichmannii* in the absence of reducing agent 0.91 (aseptic addition), and 0.17 (autoclaved with the medium), while vit. B_{12a}, which appears to be formed from vit. B_{12b} by heating at 100° for 2 hours and perhaps may be an "anhydro-form" of vit. B_{12b} which reverts on standing in solution, behaved similarly to vit. B_{12b}.

In the present investigation it was found that the potency of vit. B_{12} for *L. leichmannii* 4797 was less than that of vit. B_{12b} when both substances were assayed by aseptic addition to the previously autoclaved culture medium. However, when vit. B_{12} was autoclaved with the culture medium, its apparent potency was found to be greater than that of either vit. B_{12b} or of aseptically-added vit. B_{12}. The experimental basis of these observations was examined.

Experimental. Vit. B_{12b} was prepared by Mr. J. V. Pierce from *Streptomyces aureofaciens* as described previously(1). Solutions in water were standardized by measurement of the absorption spectrum, $E^{1\%}_{1cm}$ at 351 $m\mu = 167$. Vit. B_{12} was purchased from Merck and Co. and aqueous solutions were standardized by measurement of the absorption spectrum, $E^{1\%}_{1cm}$ at 361 $m\mu = 207$. Vit. B_{12c} (nitrosocobalamin) was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories. The concentration of the solution was not known, but was estimated to be approximately 50 γ/ml by spectrophotometric analysis using vit. B_{12b} as standard. A sample of "ammino-cobalamin" (Compound AC2) was kindly furnished by Dr. V. Petrow of British Drug Houses. Thiomalic acid, obtained from Allied Chemical and Drug Co., was recrystallized twice from water before use in the culture medium.

Microbiological assay technic. The microbiological assay technic employed in the present investigation was that of Skeggs *et al.*(5) using *Lactobacillus leichmannii* 4797. The only modification of the method was that a final volume of 2 ml of culture medium was employed rather than 10 ml; separate experiments showed this modification did not alter the experimental results. Assay results were obtained by estimating growth turbidimetrically after a 24-hour incubation at 37°C.

Results. In an attempt to compare vit. B_{12b} with vit. B_{12}, the technic of assaying the two vitamins by aseptic addition to the medium was reinvestigated. This was accomplished by diluting concentrated solutions of the vitamin by aseptic technic into sterile water and finally adding the appropriate dilution aseptically to the assay tubes containing autoclaved, cooled medium. No trouble with contamination was experienced with this technic. It was repeatedly found that the growth response of *L. leichmannii* was always less when vit. B_{12} was added aseptically to the medium as compared to when vit. B_{12} was autoclaved in the medium. Typical results are shown in Table I. In contrast liver extract, vit. B_{12b}, B_{12c} and ammino-cobalamin gave essentially the same growth response when added to the culture medium aseptically as when autoclaved. As a consequence when these samples were assayed against vit. B_{12} using aseptic technic, erroneously high results were obtained. If the samples and vit. B_{12} standard were autoclaved separately from the medium, or if sterilization of the assay was accomplished with ethylene oxide at reduced temperatures(6), results were obtained similar to those described with the aseptically-added samples in Table I. Also, if tomato juice or asparagus juice were added to the assay medium in amounts commonly employed in other vit. B_{12} microbiological assays, these additions did not alter the experimental

---

4. Kaczka, E. A., Denkewalter, R. G., Holland, A., and Folkers, K., *J. Am. Chem. Soc.*, 1951, v73, 335.

5. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

6. Wilson, A. T., and Bruno, P., *J. Exp. Med.*, 1950, v91, 449.

TABLE I. Microbiological Assay of Vitamin B_{12} with *Lactobacillus leichmannii* Comparing Aseptic and Non-Aseptic Assay Technic.

	Sample autoclaved with medium		Sample added aseptically to sterile, cooled medium	
Additions to 2 ml medium deficient in vit. B_{12}	Optical density	γ B_{12}/ml found	Optical density	γ B_{12}/ml found
0	.0		.0	
.01 mγ vit. B_{12}	.08		.08	
.03 ” ” ”	.24		.12	
.10 ” ” ”	.51		.20	
.3 ” ” ”	.62		.33	
1.0 ” ” ”	.62		.46	
3.0 ” ” ”	.62		.56	
Vit. B_{12b}, 10 γ/ml				
6 × 10^{-6} ml	.21	4.3	.24	27.0
20 × 10^{-6} ml	.45		.40	
Ammino cobalamin, 30.5 γ ml				
1.5 × 10^{-6} ml	.23	19.4	.25	150
5 × 10^{-6} ml	.49		.45	
Vit. B_{12c} sol. (ca 50 γ ml)				
0.6 × 10^{-6} ml	.19	40	.21	195
2 × 10^{-6} ml	.45		.37	
15 unit liver extr.				
1.5 × 10^{-6} ml	.30	26	.33	220
5 × 10^{-6} ml	.58		.47	
Vit. B_{12}, thiomalic-acid-reaction-product* equivalent to 2 γ vit. B_{12}/ml				
15 × 10^{-6} ml	.20	1.9	.26	13.2
50 × 10^{-6} ml	.47		.42	

* See text for details of preparation.

results shown in Table I.

The poor microbiological response to vit. B_{12} obtained when the vitamin is added aseptically to the medium suggested that when vit. B_{12} is autoclaved in the medium it may be converted to another and more active form of the vitamin. Because of the prominent role of reducing agents in microbiological assays for vit. B_{12}, the effect of heating vit. B_{12} with thiomalic acid was investigated. Fifty milligrams of thiomalic acid was dissolved in 4 ml water and the pH adjusted to 6.5; 10 γ of a solution of crystalline vit. B_{12} was added to the thiomalic acid solution, the volume adjusted to 5.0 ml, and the solution autoclaved for 10 minutes at 15 lb pressure. When this preparation was compared microbiologically with untreated vit. B_{12} it was found (Table I) to give essentially the same growth response when assayed aseptically as when autoclaved with the medium. Thus microbiologically the vit. B_{12} compound formed by heating vit. B_{12} and thiomalic acid differs from untreated vit. B_{12}. Further evidence that vit. B_{12} is transformed into a different compound when heated with thiomalic acid was obtained by paper chromatography.

The technic for chromatography followed that described by Woodruff and Foster(7). Filter paper strips buffered with 0.66M KH_2PO_4 were employed and n-butanol saturated with water used as a developer. The descending method of paper strip chromatography was used, and the chromatograms were run at 37°C for 48 hours. The position of the vitamins on the paper strips was determined bioautographically. Results of a typical experiment are given in Table II. As reported by others(7) solutions of vit. B_{12} following paper strip chromatography exhibited two zones of activity, one in the position occupied by vit. B_{12b} (35 mm) and another more rapidly-moving component characteristic of pure vit. B_{12} (180 mm). Of great interest was the finding that the preparation made by auto-

---

7. Woodruff, H. B., and Foster, J. C., *J. Biol. Chem.*, 1950, v183, 569.

TABLE II. Chromatographic Evidence for the Transformation of Vit. B_{12} to an Unidentified Compound Following Treatment with Thiomalic Acid.

Substance chromatographed	Zone	Distance from origin,† mm
Vit. B_{12}	1	35
	2	180
Vit. B_{12b}	1	35
Reaction product obtained by autoclaving vit. B_{12} and thiomalic acid*	1	60

* See text for details of preparation.

† Results are recorded as distance the vitamin moved on the paper strip from the origin rather than R_f values, as the developing solvent was allowed to drip off the end of the paper during the extended period of time employed to develop the chromatogram.

claving vit. B_{12} and thiomalic acid was chromatographically different from pure vit. B_{12}, as the reaction product moved only one-third as rapidly as vit. B_{12} but somewhat faster than vit. B_{12b}. Similar results were obtained by autoclaving vit. B_{12} in the complete assay medium containing either thiomalic acid or thioglycolic acid. From the results of Tables I and II it may be concluded that when vit. B_{12} is autoclaved in the medium in the presence of thiomalic acid it is transformed to a new compound having greater microbiological activity than vit. B_{12}, when compared by aseptic assay technic.

Earlier work from this laboratory(8) had suggested that thioglycolic acid and various reducing agents protected vit. B_{12} in crude materials from heat destruction during autoclaving. The present results indicate that the reducing agent might exert its favorable effect on growth by participating in a chemical reaction with vit. B_{12} to form a compound more readily utilized by *L. leichmannii*. To test these hypotheses further, the experiment outlined in Table III was carried out. In the absence of thiomalic acid a very poor growth response to the test samples was obtained. However, if after autoclaving the samples in the absence of reducing agent, thiomalic acid was added to the tubes and the samples reautoclaved, vit. B_{12} now exhibited essentially full activity demonstrating conclusively that the vitamin was not destroyed during autoclaving but had to be reheated with thiomalic acid to produce a good growth response. It was of particular interest that vit. B_{12b} (and liver extract) when autoclaved in the medium in the absence of thiomalic acid, gave only a slight growth response, as the data of Table I indicate that these preparations gave a good growth response when added aseptically to the medium. It is possible that heating vit. B_{12b} in the medium without thiomalic acid converts the vitamin to an inactive form which can be partially reactivated by reheating the samples with thiomalic acid. Welch and Wilson(9) have noted that thermal inactivation of vit. B_{12} may occur yielding products of which a portion can be converted by reducing agents to microbiologically more active forms.

Discussion. The results indicate that under the experimental conditions described vit. B_{12} was transformed into a new compound when it was autoclaved with the culture medium. The new compound appeared to be about five times as potent as vit. B_{12} when compared by aseptic addition. The implications of this finding in the evaluation of the vit. B_{12} content of natural materials are important. If vit. B_{12}, autoclaved with the medium, is used for the construction of the "standard curve," then a somewhat low value may be obtained for the potency of samples which contain predominantly vit. B_{12b}. An erroneously high value for such samples may be obtained if the standard curve is constructed from values obtained with vit. B_{12} added aseptically. If solutions of vit. B_{12} are inadvertently exposed to light, the resultant decomposition to form vit. B_{12b} may complicate the microbiological findings.

Two possibilities suggest themselves for resolving these discrepancies: (I) treatment of the samples with cyanide to convert vit. B_{12b} into vit. B_{12}(10-12) and use of vit. B_{12} as a standard, (II) photolysis of the samples if vit. B_{12} is present to convert it into vit. B_{12b}

---

8. Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffmann, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, v8, 257.

9. Welch, A. D., and Wilson, M. F., *Arch. Biochem.*, 1949, v22, 486.

TABLE III. Effects of Thiomalic Acid (TMA) on the Microbiological Assay of Vitamin B_{12}.

Addition to 2 ml medium deficient in vit. B_{12}	Samples autoclaved in medium containing		
		No TMA	
	1 mg TMA per tube		Following autoclaving 1 mg TMA added/tube and assay reautoclaved
	Optical density		
0	.02	.02	.02
.03 mγ vit. B_{12}	.30	.04	.22
.08 ” ” ”	.57	.10	.52
.27 ” ” ”	.78	.27	.80
.1 mγ vit. B_{12b}	.38	.03	.14
.3 ” ” ”	.63	.06	.29
1.0 ” ” ”	.84	.16	.66
.67 × 10^{-6} ml liver extract	.19	.01	.09
2.0 ” ” ” ” ”	.39	.03	.17
6.66 ” ” ” ” ”	.69	.06	.35

(10) or perhaps standing in dilute HCl(13) to convert to a vit. B_{12b}-like compound, and use of vit. B_{12b} as a standard. These possibilities are being investigated.

Vit. B_{12c} and "ammino-cobalamin" both appeared, like vit. B_{12b}, to give the same growth response when the substance autoclaved with the medium was compared with the aseptically-added substance. The results obtained in patients with pernicious anemia (2,14,15) did not indicate a quantitative difference in clinical potency between vit. B_{12} and B_{12b}. The two compounds appear to have the same potency when injected into chicks (1). The potency of both vit. B_{12} and B_{12a} for *L. lactis* was enhanced by autoclaving with the culture medium(16). The addition of thioglycolic acid to the culture medium further increased the potency of both substances and their activity became equal. The results in their Fig. 1 indicate that the potency of vit. B_{12} for *L. leichmannii* was increased somewhat by autoclaving with the culture medium as compared with the potency of the vitamin when added aseptically. This increase did not appear to have been observed for vit. B_{12a} and B_{12b}.

Summary. 1. A comparison was made of vit. B_{12}, B_{12b}, B_{12c} and "ammino-cobalamin" in the microbiological assay with *Lactobacillus leichmannii* using aseptic or non-aseptic assay technic. The growth response of *L. leichmannii* was significantly less when vit. B_{12} was added aseptically to the medium as compared to when vit. B_{12} was autoclaved in the medium. This effect was not noted for vit. B_{12b}, B_{12c} and "ammino-cobalamin." 2. By means of paper strip chromatography it was demonstrated that when vit. B_{12} is autoclaved with thiomalic acid, vit. B_{12} is transformed into a new compound which is distinct from vit. B_{12b}. The vit. B_{12}-thiomalic acid reaction product is more potent microbiologically than vit. B_{12} when the compounds are compared by aseptic assay technic. 3. The significance of these results in the interpretation of the microbiological assay of the vit. B_{12} content of natural materials when vit. B_{12}

---

10. Veer, W. L. C., Edelhausen, J. H., Wijmenga, H. G., and Lens, J., *Biochimica et Biophysica Acta,* 1950, v6, 225.

11. Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., and Folkers, K., *Science,* 1950, v112, 354.

12. Beaven, C. H., Holiday, E. R., Johnson, E. A., Ellis, B., and Petrow, V., *J. Pharm. and Pharmacol.,* 1950, v2, 12.

13. Brockman, J. A., Jr., Pierce, J. V., Stokstad, E. L. R., Broquist, H. P., and Jukes, T. H., 117th ACS meeting, Philadelphia, April 10-13, 1950; Abstracts of Papers 11A.

14. Lichtman, H., Watson, J., Ginsberg, V., Pierce, J. V., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 643.

15. Spies, T. D., Stone, R. E., Koch, M. B, Grant, H. M., and Moore, M. M., *S. Med. J.,* 1950, v43, 50.

16. Hendlin, D., and Soars, M. H., *J. Biol. Chem.,* 1951, v188, 603.

is used as a standard is briefly discussed.

Addendum. During the progress of this work we received a copy of a paper by H. W. Long and O. L. Kline(17) in which, from studies similar to ours, it was postulated that both vit. B_{12} and vit. B_{12b} following heating in the basal medium are converted to a form of vit. B_{12} more readily utilizable by *L. leichmannii.*

The assistance of Mrs. Lucy Smith is gratefully acknowledged.

---

17. Long, H. W., and Kline, O. L., *J. Assn. Official Ag. Chemists,* in press.

Received March 28, 1951. P.S.E.B.M., 1951, v76.

Estrogen-Androgen Antagonism: Histology of Mammary Glands and Vaginal Grafts of Male Mice Receiving Estrogens.* (18640)

Stuart W. Arhelger† and Robert A. Huseby.

From the Departments of Surgery, and of Physiology, Division of Cancer Biology, University of Minnesota Medical School.

The concept of an essential antagonism between estrogens and androgens has been the subject of much endocrinological investigation and considerable debate. In the early endocrine literature, Steinach and Kun(1), Lipschutz and others(2,3) refer to what they considered to be antagonistic actions between the gonads of the opposite sexes and later applied this concept to the sex hormones as well. However, extensive work culminating in the classic publication of Moore and Price (4), refuted this hypothesis, contending that gonad hormones stimulate homologous reproductive accessories but are without direct effect upon heterologous accessories and have no direct effect on the gonads of either the same or opposite sex, gonadal activity being governed by the function of the pituitary gland. Although most instances of "hormonal antagonism" observed prior to 1932 probably were effected through the suppression of pituitary function, several more recent reports seem clearly to demonstrate the existence of both cooperative and antagonistic interactions between estrogenic, androgenic and progestational hormones(5-16). Although there are certain differences in the usage of the terms hormone "antagonism" and "cooperation", it would seem best to restrict the term "antagonism" to those situations in which one hormone reduces the magnitude of the response of a target tissue or end organ to another hormone. To demonstrate such an-

---

* Assisted by grants from the Minnesota Division of the Am. Cancer Soc., and the Am. Cancer Soc. upon recommendation of the Committee on Growth of the National Research Council.

† Clinical Fellow in Surgery, Am. Cancer Soc.

1. Steinach, E., and Kun, H., *Biol. Gen.,* 1926, v2, 815.

2. Lipschutz, A., *Endocrinology,* 1925, v9, 109.

3. Lipschutz, A., and Vinals, E., *C. R. Soc. Biol.,* 1927, v97, 1400.

4. Moore, C., and Price, D., *Am. J. Anat.,* 1932, v50, 13.

5. Korenchevsky, V., *Nature,* 1936, v137, 494.

6. Korenchevsky, V., and Hall, K., *J. Path. and Bact.,* 1937, v45, 681.

7. Deansly, R., and Parkes, A., *Quart. J. Exp. Physiol.,* 1936-37, v26, 394.

8. Mühlbock, O., *Proc. Internat. Cong. Obstet. and Gynec.,* 1938, v2, 491.

9. Mühlbock, O., *Acta Brevia Neerlandica,* 1938, v8, 50.

10. Gardner, W., and Pfeiffer, C., Proc. Soc. Exp. Biol. and Med., 1938, v38, 599.

11. Shorr, E., Papanicolaou, G., and Stimmel, F., Proc. Soc. Exp. Biol. and Med., 1938, v38, 759.

12. Huggins, C., and Clark, P., *J. Exp. Med.,* 1940, v72, 747.

13. Mühlbock, O., *Acta Brevia Neerlandica,* 1942, v12, 85.

14. Albert, S., *Endocrinology,* 1942, v30, 454.

15. Smith, O., and Smith, G., Proc. Soc. Exp. Biol. and Med., 1944, v57, 198.

16. Mühlbock, O., *Acta Brevia Neerlandica,* 1948, v16, 1.

tagonism it would seem desirable, if not essential, to arrange the hormone doses in such a way that the stimulating effects of one hormone are completely inhibited or abolished by the antagonizing hormone. If, however, the response of a target tissue is altered in a qualitative manner but not inhibited by the addition of a second hormone, "cooperative actions" may be said to exist.

The present study was prompted by the demonstration in mice that testis function will inhibit the stimulating effects of estrone for both the glandular epithelium of the mammae (16,17) and the epithelial lining of vaginal grafts(17). Evidence that this represents an estrogen-androgen antagonism is suggested by the work of both Mühlbock(16) and Gardner(18), who noted that the administration of large doses of testosterone propionate counteracted the proliferative influence exercised by injections of estrone and estradiol benzoate on the development of the mammary glands of male mice. The purpose of this investigation is to extend the observations concerning the action of testicular function in inhibiting mammary gland and vaginal mucosal proliferation induced by steroid estrogens, and to ascertain if a similar antagonism exists between testes function and the actions of a non-steroid estrogen such as diethylstilbestrol.

Materials and methods. Closely graded doses of two steroid estrogens (estrone and estradiol[‡]) and one stilbene estrogen (diethylstilbestrol) were administered to groups of castrate and non-castrate male mice bearing subcutaneous vaginal grafts. It has been shown in a previous investigation(17) that the epithelial lining of such vaginal grafts present essentially the same histology as normal vaginal mucosa and will undergo the same histological changes when subjected to hormonal stimulation. Intact testes were chosen as the source of androgen because of the extensive and contradictory literature dealing with the possible "estrogenic actions" of injected androgens on the mammary gland and vaginal mucosa of female mice(7,19-21). Shortly after weaning, male mice of the F_1 hybrid cross between the A and Z stocks[§] received implants of vaginal tissue in the deep subcutaneous tissues of the groin, the donors being female mice of the same age and strain. Castrations were carried out after the grafts had been allowed to vascularize for 4 weeks and hormone injections were begun the following day. The estrogenic substances were administered in 10% alcohol-aqueous solutions in 2 daily injections of 0.2 cc each. Injections were continued for 21 days with the exception of one series that received estrone .04 γ per day 4 days of the week for 3 weeks. All animals were sacrificed within 6 hours of the last injection. A total of 196 mice divided into 27 series were studied. The mammary glands were studied by the whole mount technic. Multiple sections of the vaginal grafts were examined microscopically and the accessory sex organs were examined grossly and in certain groups microscopically in order to estimate testicular function (to rule out evidence of pituitary suppression).

Results. The mammary glands of the uninjected control mice revealed no evidence of stimulation and consisted of only a few narrow branching ducts without the formation of end buds. The mucosa of the vaginal grafts in these animals was atrophic and consisted of a double layer of low, cuboidal cells, similar to the vaginal mucosa of recently ovariectomized female mice. It would therefore appear that testicular function alone does not alter the histology of this mucosa.

In order to be certain that the estrogenic substances injected did not cause significant pituitary suppression, resulting in reduction of testicular function, the accessory sex organs

---

17. Huseby, R., and Bittner, J., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 321.

18. Gardner, W., *Recent Progress in Hormone Research,* 1947, p. 234 (Academic Press, Inc., New York, N. Y.).

[‡] The estrone and estradiol were kindly supplied by Schering Corporation.

19. Leonard, S., and Reece, R., *Endocrinology,* 1942, v30, 32.

20. McEven, C., Selye, H., and Collip, J., PROC. SOC. EXP. BIOL. AND MED., 1936, v35, 56.

21. Von Heuverswyn, J., Folley, S., and Gardner, W., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 389.

[§] Supplied through the courtesy of Dr. J. J. Bittner.

TABLE I. Male Mice Bearing Vaginal Grafts Injected with Estrone.*

γ per day		Vaginal mucosa		Mammary gland development	
.04 γ 4 days/wk	Castrate	Cornification and mucification	(4)	0 Slight	(8) (1)
	Non-castrate	Atrophic	(5)	0	(10)
	Adrenalectomized castrate	Cornification	(4)	Good	(4)
	Adrenalectomized non-castrate	Atrophic	(5)	0	(5)
.05	Castrate	Cornification and mucification	(5)	Good	(5)
	Non-castrate	Development—almost complete mucification	(5)	0	(4)
.10	Castrate			Good	(6)
	Non-castrate			0	(5)
.15	Castrate	Complete cornification	(7)	Good	(9)
	Non-castrate	Almost complete cornification	(4)	0 Slight	(3) (1)
Uninj. controls	Non-castrate	Atrophic	(18)	0	(18)

* The number in parentheses indicates number of mice in each group.

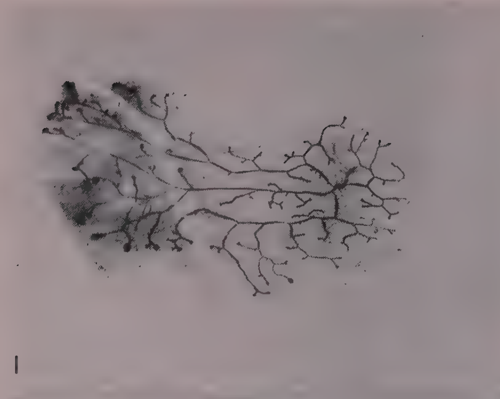
of the animals were carefully studied, especially in the groups receiving the higher estrogen doses. The accessory sex organs of the animals were found to be normally developed in all of the non-castrate animals. This suggests that relatively little or no pituitary suppression occurred under the conditions of these experiments. It is also of some interest to record that in the castrate males no evidence of squamous metaplasia of the seminal vesicular or prostatic epithelia occurred with the relatively small doses of estrogens employed in these studies.

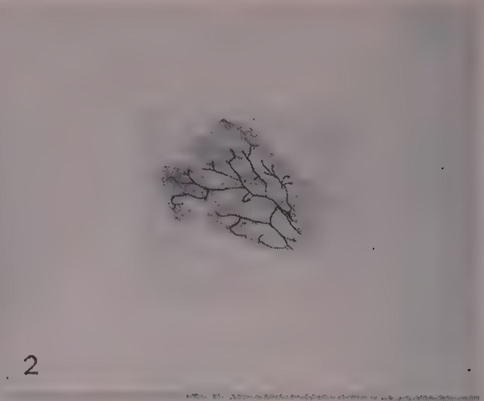
Estrone series (Table I). Evidence for androgenic antagonism to the proliferation of the mammary gland induced by a steroid estrogen was noted at the .05 γ dose of estrone¶ and was exhibited through the .15 γ dose level. At these dose levels there was good development present in all of the castrate animals, (all mammary glands contained numerous club-shaped swellings at the end of lactiferous ducts with numerous mitotic figures), while the glands of all the non-castrate animals receiving the .05 γ and the .10 γ doses and 3 of the 4 glands of the non-castrate animals given the .15 γ dose were without development. (Fig. 1 and 2). Similar antagonism for the vaginal mucosa was observed at the .04 γ dose of estrone when administered 4 times per week. In the castrate group at this dose level there was definite development in the form of cornification and mucification while there was no histological evidence of stimulation in the non-castrate group. (Fig. 3 and 4).

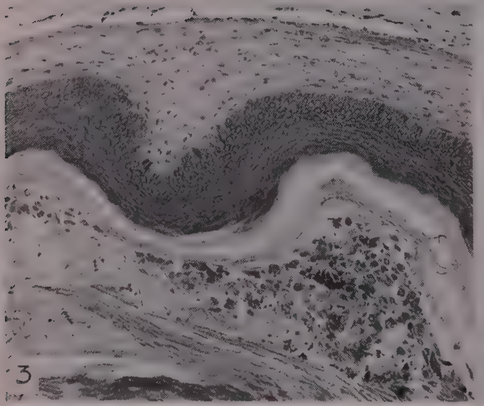
It is apparent from the study of the vaginal epithelium that antagonism, when it occurs, is exhibited within a rather narrow range of dosage. As the dose of estrone is increased above this critical range, the antagonism appears to be overcome and then as, at the .05 γ level, the cooperative effects of the hormones, as evidenced by epithelial mucification, become apparent, while at still higher doses, for example .15 γ, neither antagonism nor cooperation is evident and the epithelium is completely cornified reflecting only the estrogenic stimulation. In many strains of mice following gonadectomy the adrenal cortex has been shown to produce rather large amounts of estrogenic hormones. Therefore, an experiment was carried out to ascertain if the differences noted between the non-castrate and castrate groups of animals

---

¶ In an earlier study(17) the mammae of castrate male mice of this hybrid cross receiving .05 γ estrone per day were reported as not being developed because at that time we considered both end bud formation and definite duct extension as being necessary criteria of development. A review of these earlier slides reveals that the glands of all 4 of the animals contained several end buds and should have been recorded as showing development.







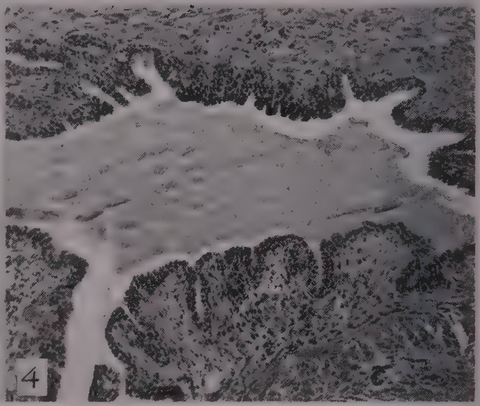


FIG. 1. Mammary gland of a castrate male mouse receiving .10 γ estrone per day. Note the presence of numerous end buds, and the definite increase in the size of the gland that has occurred.

FIG. 2. Mammary gland of a non-castrate male mouse receiving .10 γ estrone per day. No end buds are present and the gland is similar to those seen in uninjected control male mice of this stock.

FIG. 3. Vaginal graft of a castrate male mouse receiving .04 γ estrone 4 days of the week. The epithelium is almost completely cornified.

FIG. 4. Vaginal graft of a non-castrate male mouse receiving .04 γ estrone 4 days of the week. No evidence of epithelial stimulation is seen and the graft is similar to those seen in uninjected control male mice.

could be due to differences in adrenal function.‖ The result of this study has been included in Table I. It can be seen that the differences between the castrate and non-castrate mice are still present after adrenalectomy. In fact, the castrate adrenalectomized mice appear somewhat more sensitive to estrone than do the castrate non-adrenalectomized animals.

Estradiol series (Table II). Similar evidence for androgenic antagonism to the proliferation of the mammary gland occurred at the .01 γ per day and .02 γ per day doses of estradiol. At the .01 γ per day dose level, there was development present in the glands of 8 of the 9 castrate animals, while the glands

---

‖ Month-old mice were castrated and adrenalectomized and then were maintained on sodium chloride and glucose drinking water and a semi-synthetic diet in which sodium salts had been substituted for the potassium salts in the salt mix (graciously supplied by Dr. J. T. King). Studies have indicated that on this regimen young adrenalectomized mice grow well and show essentially normal development of their secondary sex characteristics.

TABLE II. Male Mice Bearing Vaginal Grafts Injected with Estradiol.*

γ per day		Vaginal mucosa		Mammary gland development	
.0025	Castrate	Atrophic	(4)	Slight	(1)
				0	(4)
	Non-castrate	”	(3)	0	(5)
.01	Castrate	Cornification	(2)	Good	(7)
		Cornification and mucification	(1)	Slight	(1)
				0	(1)
	Non-castrate	Atrophic	(3)	0	(8)
		Slight mucification	(1)	Slight	(1)
.02	Castrate			Good	(8)
	Non-castrate			0	(9)

* The number in parentheses indicates number of mice in each group.

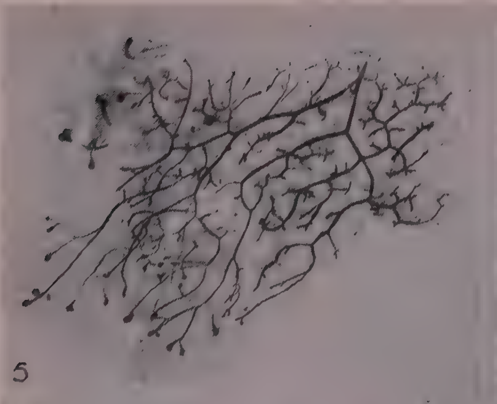
TABLE III. Male Mice Bearing Vaginal Grafts Injected with Diethylstilbestrol.

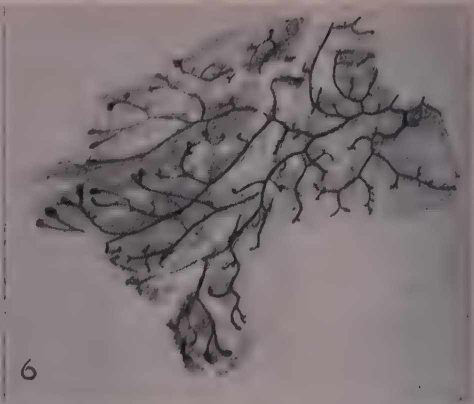
γ per day		Vaginal mucosa		Mammary gland development	
.004	Castrate	Atrophic	(5)	0	(6)
				Slight	(1)
	Non-castrate	”	(4)	0	(3)
				Slight	(2)
.008	Castrate	Slight development	(9)	0	(12)
		Some mucification		Slight	(2)
	Non-castrate	Slight development	(9)	0	(8)
		Some mucification		Slight	(1)
.016	Castrate	Mucification with beginning cornification	(9)	Good	(9)
	Non-castrate	Mucification	(9)	”	(9)
.033	Castrate	Almost complete cornification	(5)	”	(5)
	Non-castrate	Almost complete cornification	(5)	”	(5)
.066	Castrate	Complete cornification	(4)	”	(4)
	Non-castrate	” ”	(4)	”	(4)

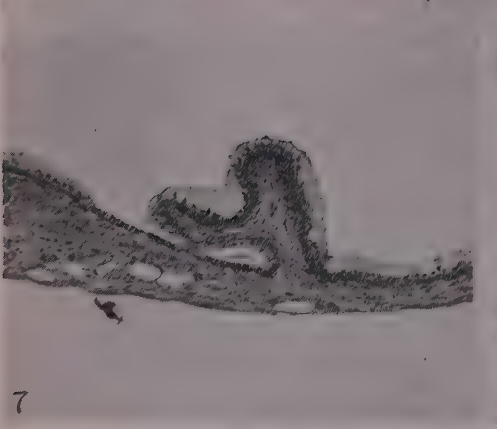
* The number in parentheses indicates number of mice in each group.

of 8 out of 9 of the non-castrate animals were without development (one gland of one non-castrate animal contained 2 end buds). The glands of all of the castrate animals receiving .02 γ per day were well-developed in contrast to a complete absence of development in all of the non-castrate animals receiving the .02 γ per day dose. Evidence for androgenic antagonism to the development and cornification of the vaginal mucosal grafts was present also at the .01 γ per day dose level of estradiol. All of the grafts in castrate animals were cornified (a small amount of mucification accompanied the cornification in two cases) whereas all but one of the grafts in non-castrate animals remained completely atrophic. A slight amount of mucification was present in this one graft.

Diethylstilbestrol series (Table III). Evidence of estrogen-androgen antagonism seemed to be lacking when the non-steroid diethylstilbestrol was the estrogenic substance administered. In contrast with the estrone and estradiol series there was a striking similarity when the mammary gland and vaginal graft histology of the castrate animals was compared with that of the non-castrated animals receiving the same doses of diethylstilbestrol (Fig. 5, 6, 7, 8). This varied from no development in the .004 γ per day dose to complete development of both epithelia at the .066 γ per day dose. The only appreciable differences demonstrable between the castrate and non-castrate animals occurred in the vaginal mucosa at the .016 γ per day dose. The grafts of these castrate animals revealed mucification with beginning cornification, while only mucification was present in the







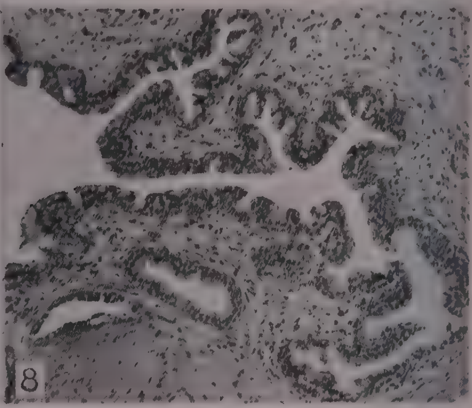


FIG. 5. Mammary gland of a castrate male mouse receiving .033 γ diethylstilbestrol per day. Several end buds are to be seen and the duct system has extended.

FIG. 6. Mammary gland of a non-castrate male mouse receiving .033 γ diethylstilbestrol per day. This gland is similar to those seen in the castrate male mice receiving the same dose of diethylstilbestrol.

FIG. 7. Vaginal graft of a castrate male mouse receiving .008 γ diethylstilbestrol per day. There is evidence of a minimal stimulation consisting only of mucification of the luminal cells.

FIG. 8. Vaginal graft of a non-castrate male mouse receiving .008 γ diethylstilbestrol per day. The degree of stimulation is similar to that seen in the grafts of the castrate males receiving this dose of diethylstilbestrol.

non-castrate animals. This is interpreted as indicating that with functioning testes cooperative effects were evident over a wider range of injected estrogen.

Discussion. The presence of an estrogen-androgen antagonism seems fairly well established by the results of investigations with estrone and estradiol. It is evident that testicular function is capable of completely inhibiting the stimulating effects of certain doses of both of these steroid estrogens on the mammary and vaginal epithelia. That this antagonism is due to testicular androgen is supported by the studies of Mühlbock and Gardner(16,18) in which the injection of relatively large doses of testosterone propionate inhibited the mammary stimulating effects of estrone and estradiol.

The apparent lack of pituitary suppression as evidenced by the normal testicular stimulation of the accessory sex organs of the non-castrate animals (in all series regardless of estrogen dosage) strongly supports the concept of estrogen-androgen antagonism as a direct or essential phenomenon for certain target tissues. It seems probable that in many experiments in the past, such as those reported by Moore and Price(4), antagonistic or neutralizing effects were not observed due to the use of relatively large doses of the stimulating hormones. In our study of the vaginal epithelium, if only the 0.15 γ per day or larger doses of estrone had been employed no inhibitory or cooperative effects of testicular function would have been observed. If instead of graded doses, a single low level such as 0.05 γ had been employed, only the cooperative effects would have been observed in the target tissue.

Of particular interest is the apparent lack of estrogen-androgen antagonism in the studies with diethylstilbestrol. Although an explanation of the mechanism for hormone antagonism must await further experimental data, the interactions of the naturally occurring estrogens and androgens may possibly represent another instance of biological competition occurring between structurally related compounds similar to the competitive inhibition exhibited between the metabolite thiamine and its structural analogue pyrithiamine(22). One might hypothecate further that the lack of such competitive inhibition between diethylstilbestrol and testicular androgens might suggest that the androgens are not able to successfully compete with diethylstilbestrol for some particular intermediary substance, possibly an enzyme, whereas they can successfully compete for this substance with estrone or estradiol. This difference in action would also result if the metabolic pathways for the utilization of, or the stimulation by, the two types of estrogens were somewhat different so that the step at which androgens inhibit or block the steroid estrogens is not involved in diethylstilbestrol stimulation of these target organs.

Summary. 1. Studies of the effect of injections of steroid and non-steroid estrogenic substances on the mammary gland and vaginal mucosa of castrate and non-castrate male mice bearing subcutaneous vaginal grafts are reported. 2. It is suggested that antagonistic actions exist between the naturally-occurring estrogens (estrone, estradiol) and testicular androgens in regard to the mammary gland and vaginal mucosa. 3. Such antagonism did not seem to occur when the synthetic estrogenic substance diethylstilbestrol was employed.

---

22. Woolley, D., *Physiol. Rev.*, 1947, v27, 308.

Received December 27, 1950. P.S.E.B.M., 1951, v76.

Propagation in Chick Embryos of Dengue Virus, Hawaiian Strain. II. Findings in Infected Eggs.* (18641)

R. WALTER SCHLESINGER.

From the Division of Infectious Diseases, Public Health Research Institute, City of New York.

It has been reported(1) that propagation of the Hawaiian strain of dengue virus was facilitated by progressive adaptation of the virus to mice by means of intracerebral passages. After 101 such passages, the virus was transferred to chick embryos, and it has to date been carried through more than 90 passages in eggs without demonstrable changes in its pathogenicity for mice and without change in its limited host range. The sera of human beings and of monkeys convalescent from infection with the original, human Hawaiian strain react specifically with the egg-adapted virus. Of two human volunteers inoculated with it, only one had a mild febrile reaction, but both responded with production of neutralizing antibody against the mouse-adapted virus(1).

This paper will describe certain characteristics of the infection in eggs. Of main interest is the finding that the pronounced neurotropism, acquired by the virus as a result of passage through mice, has persisted in the

---

* This investigation was supported in part by a research grant from the National Institutes of Health, U. S. Public Health Service.

1. Schlesinger, R. W., *Am. J. Hyg.*, 1950, v51, 248.

TABLE I. Effect of Age of Eggs at Time of Inoculation on Concentration of Virus After 7 Days' Incubation.

Inoculum	Age of embryos at inoculation (days)	Mortality rates among mice inoc with embryo dilution						Log LD_{50}*
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
M101-E88	4	5/5	5/5	5/5	4/4	2/5	0/5	4.8
	5	5/5	5/5	5/5	4/5	3/5	0/5	5.0
M101-E15	5	5/5	5/5	4/4	3/5	3/5	1/5	5.0
	7	5/5	5/5	5/5	2/5	0/5	0/5	3.8
	9	4/4	4/5	1/5	0/5	0/5	0/5	2.5

* Log LD_{50} expressed as reciprocal of highest dilution estimated to kill 50% of the mice.

infection of chick embryos.

Materials and methods. The materials and methods used in this work have been described in the preceding paper of this series(1). The virus samples used were from egg passages which will be referred to below as "M101-E (n)." Unless otherwise mentioned, the test materials were 50% suspensions of whole embryos prepared as described previously(1). Dilutions were made in saline containing 1/10 volume of heated (56°C for 30 minutes), Seitz-filtered normal rabbit serum and 500 units per ml of sodium penicillin G. The inoculum consisted of 0.1 ml which was deposited in the neighborhood of the embryo with a 1.5 inch needle which was inserted through the air sac ("peri-embryonic stab") (2). The infectivity of materials harvested from eggs was determined by intracerebral subinoculation in mice. 0.03 ml of the test sample in suitable concentration was the inoculum. All titers have been expressed in terms of LD_{50} per 0.03 g of tissue or ml of fluid.

Experimental. Factors affecting the yield of virus in chick embryos. A. Age of embryos at time of inoculation. When the age of embryos at the time of inoculation was varied, results such as shown in Table I were obtained. All of the embryos were harvested after seven days' secondary incubation. The yield of virus was comparable in eggs inoculated on the fourth or fifth day of incubation, but inoculation on the seventh or ninth day resulted in reduced titers. This indicated a trend similar to that shown for the mouse-adapted Hawaiian virus which multiplies to higher titers in baby than in adult mice(3). A single attempt was made to see if further reduction in age of chick embryos at the time of inoculation might further enhance the titer. Two-day-old embryos were inoculated through a hole in the vitelline membrane, according to the method used by Hamburger and Habel in experiments with influenza and mumps viruses (4). The yield of virus in the embryos surviving after 7 days' secondary incubation was not significantly different from that obtained in 5-day-old embryos.

B. Period of secondary incubation. When the present series in eggs was initiated, it was found(1) that serial passages at 5-day intervals led to progressive decrease in viral concentration so that no virus was detectable beyond the sixth passage. On the other hand, with egg-to-egg transfers done every 7 days, the series could be maintained satisfactorily. After the virus had been propagated in this manner through a number of passages, its rate of multiplication in eggs increased so that infective titers of embryos reached maximum after 5 days, and remained at the highest level through the seventh day. Embryos harvested later, *i.e.* after intervals of up to 12 days after inoculation, contained only slightly reduced amounts of virus. Thus, 4 days before normal hatching time, the titer remained at levels of 10^{-3} to 10^{-4}.

C. Temperature of incubation. Eggs inoculated with embryo suspension from passage M101-E52 were incubated for 7 days at 35 or 39°C. The infective titers of the embryos of

---

2. Fox, J. P., and Laemmert, H. W., *Am. J. Hyg.*, 1947, v46, 21.

3. Sabin, A. B., and Young, I., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 478.

4. Hamburger, V., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1948, v66, 608.

TABLE II. Comparative Susceptibility of Mice and Chick Embryos to Egg-Adapted Hawaiian Dengue Virus. (Passage M101-E29).

	Dilution of embryo suspension inoculated					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
No. of mice dead after inoculation of indicated dilution	5/5	5/5	5/5	5/5	1/5	0/5
Log LD_{50}/0.03 g of embryo tissue 7 days after inoculation of indicated dilution	4.0	3.2	3.2	3.6	2.9	n.t.

TABLE III. Distribution of Virus in Eggs Harvested 7 Days After Inoculation.

	Log LD_{50} per .03 g or .03 ml of								
Inoculum egg passage No.	Whole embryo	CNS	Skeletal muscle	Viscera	Amniotic membrane	Amniotic fluid	Chorio-allantoic membrane	Allantoic fluid	Yolk sac
M101-E9	4.6	5.5	—	—	1.7	.6?*	.5?	0†	1.0?
M101-E11	—	4.6	<2.6	—	—	—	—	—	—
M101-E13	—	5.4	2.3	—					
M101-E23	—	4.2	2.2	—	—	—	—	—	
M101-E49	—	5.3	3.6	2.7	1.8	.5?	.8?	.3?	1.5
M101-E85	—	5.4	2.5		1.3	.6?	.7?	.4?	1.2

*.6? Question marks indicate that endpoints are rough estimates in titrations in which only a few mice died after inoculaton of undiluted materials. In the case of the chorio-allantoic membranes, the dilution factor of the original suspension is inaccurate because of admixture of allantoic fluid.

† "O" indicates failure of undiluted fluid to produce paralysis or death in any of the mice inoculated.

— Not tested.

the two groups did not differ significantly.

D. Size of seed inoculum. Several experiments were carried out to determine the amount of virus which would result in optimal yields in eggs, and to compare the susceptibility to infection of embryos with that of mice. One such experiment is summarized in Table II. Ten-fold serial dilutions of embryo suspension M101-E29 were inoculated simultaneously into mice and into 5-day-old eggs. It was found that the infectivity of the virus for eggs was at least as high as for mice, in that the 10^{-5} dilution gave rise to infection in embryos as well as in one of the 5 mice inoculated. Here, as in other similar tests, dilute seed inocula resulted in reduced rather than in increased viral yield after 7 days. There was no indication that large inocula might produce the phenomenon of "auto-interference" similar to that described for influenza virus(5).

Distribution of virus in infected eggs. Eggs inoculated by periembryonic stab with material derived from different passages were harvested after 7 days' incubation and the virus contents of various fluids and organs were assayed. As can be seen in Table III, the highest concentration of virus was found in the central nervous system. Skeletal muscles and viscera contained 100 to 1,000 times less. Some viral multiplication may have taken place in the amniotic membranes and yolk sacs, since they contained more virus than amniotic or allantoic fluids or the chorio-allantoic membrane. The low degree of susceptibility of the chorio-allantoic membrane to infection was further illustrated by the following experiment. Embryo suspension from passage M101-E17 was inoculated on the dropped chorio-allantoic membrane of seven-day-old eggs, which were then incubated at 37°C. Three eggs each were harvested after 4, 5, 6 and 7 days, and their fluids and tissues assayed for virus. On the fourth day, virus was detectable only in blood. It appeared in the embryo on the fifth day, but the titer failed to rise above 10^{-2} in any of the specimens tested. All extra-embryonic fluids and membranes remained free of demonstrable

---

5. Henle, W., and Henle, G., *Am. J. Med. Sc.*, 1944, v207, 705.

virus throughout the experimental period. The low concentration of virus attained under these conditions may be related to the time elapsed before virus reached the embryo itself when inoculated by this route (negative on the fourth day after inoculation, *i.e.* 11th day of incubation).

Effect of infection with dengue virus on the chick embryo. A. Mortality and hatchability. As a rule, infected eggs were incubated at 35°C and at this temperature even un-inoculated embryos showed poor growth, and a high proportion died at hatching time. On several occasions, small numbers of infected and normal control eggs were incubated at higher temperatures until hatching time. Thus, in one group of 26 normal eggs incubated at 38°C, only 11 hatched. Those which failed to hatch survived until hatching time but died on the 23rd to 25th day of incubation. On the other hand, of 24 eggs inoculated on the eighth day of incubation with virus (M101-E17), 21 survived until hatching time; of these 13 hatched. While the existing conditions appeared to be generally unfavorable for hatching, the hatchability did not seem to be affected by infection with dengue virus. None of the hatched normal or infected chicks survived beyond the tenth day of life. The cause of their death has not been determined.

B. Pathological findings. The gross appearance of embryos harvested from infected eggs from 5 to 12 days after inoculation showed no consistent deviation from that of normal embryos of the same ages. All harvested embryos were weighed, and there was no difference between normal and infected groups. Semi-serial cross-sections of normal and infected 13-day-old embryos were stained with gallocyanin-eosin and compared carefully at each level. Because the central nervous system supported viral growth preferentially, this organ was studied with particular care. No pathological lesions were seen. There were large numbers of mitotic figures in the CNS and in other organs of infected as well as normal embryos, suggesting that infection did not interfere with their growth and development.

Persistence of infection after hatching. It had been found that dengue virus persisted in embryos at fairly high concentrations for at least 12 days after inoculation, *i.e.* up to 4 days before normal hatching time. In view of the inability of chick embryos to respond to infection with antibody formation, it was considered of interest to see how long after hatching virus remained demonstrable. Twelve one- or 2-day-old chicks hatched from eggs inoculated on the eighth day of incubation with embryo suspension M101-E17 were bled from the heart into a syringe containing heparin. Each blood sample was immediately inoculated intracerebrally into 5 mice. As shown in Table IV, seven or eight of the specimens contained virus. However, the number of mice which became paralyzed was so small, and their mean survival time so long, that the concentration of virus in each positive sample must have been near the limits of detectability. It has been reported previously(1), and since then confirmed in numerous titrations, that a consistent correlation exists between virus concentration and mean survival time.

Because of the presence of large amounts of virus in the CNS tissue of infected embryos, it was of interest to see whether it persisted there after hatching. A total of 19 chicks from groups inoculated with 3 different virus samples died or were sacrificed at various intervals of up to 10 days after hatching. Their brains and spinal cords were removed and ground up individually. Twenty percent suspensions were inoculated into groups of mice. The results are summarized in Table V. Of 19 chicks, 17 or 18 harbored dengue virus in their CNS. The incidence of infection in the individual groups of mice, and their mean survival time, indicated that the CNS of all chicks dead or sacrificed up to 7 days after hatching or up to 20 days after inoculation contained an estimated 10 to 1,000 LD_{50}, while those tested 8 to 10 days after hatching, *i.e.* 22 days after inoculation, contained less virus or none at all. Unfortunately, as has been mentioned, attempts to raise either normal or infected chicks beyond the tenth day have thus far been unsuccessful. Further efforts in this direction will be made.

TABLE IV. Presence of Virus in Blood of Chicks Hatched from Dengue-infected Eggs. Inoculum: Passage M101-E17 embryo suspension.

Chick No.	Age of embryo at inoculation	Day of hatching	Age of chick at time of bleeding (days)	Interval betw. inoculation and bleeding (days)	Result of intracerebral inoculation in mice	No. of positive samples
1	8	21	2	15	1/5[*] (14)[†]	2/3
2					3/5 (15)	
3					0/5	
4	8	22	1	15	0/5	2/4
5					1/5 (13)	
6					0/5	
7					3/5 (13)	
8	8	23	2	17	2/5 (13.5)	3 or 4/5
9					1/4 (12)	
10					0/4	
11					1?/4[‡] (14)	
12					2/5 (13.5)	
				Total	13 or 14/58	7 or 8/12

---

[*] 1/5 No. of mice dying of dengue infection in enumerator, No. observed in denominator.
[†] Figures in parentheses indicate average time of death of paralyzed mice.
[‡] One mouse died, cause of death not ascertained.

TABLE V. Presence of Virus in CNS of Chicks Hatched from Dengue-infected Eggs.

Inoculum	Age of embryos at time of inoc. (days)	Chick No.	Day of hatching	Age of chick at time of test (days)	Interval between inoc. and harvest of CNS (days)	Result of intracerebral inoculation in mice	No. of positive samples
M101-E14	9	1	25	4	20	10/10[*] (12)[†]	1/1
		13	23	0	15	6/6 (11.2)	
		8	23	2	17	5/5 (9.8)	
		10	23	2	17	5/5 (10.8)	
		11	23	2	17	5/5 (11.8)	
		12	23	2	17	5/5 (10.0)	
		9	23	5	20	5/5 (11.0)	
M101-E17[§]	8	4	22	6	20	5/5 (11.2)	13/13
		5	22	6	20	4/5 (11.8)	
		2	21	7	20	5/5 (11.8)	
		3	21	7	20	5/5 (10.8)	
		6	22	8	22	4/5 (13.0)	
		7	22	8	22	1/5 (13.0)	
		1	21	9	22	3/5 (12.7)	
M101-E51	9	521	21–22	9–10	22	0/5	3 or 4/5
		522	21–22	9–10	22	3/4 (14.3)	
		524	21–22	9–10	22	1?/3 (12.0)[‡]	
		525	21–22	9–10	22	4/5 (11.2)	
		526	21–22	9–10	22	4/4 (12.8)	
					Total	79–80/97	17 or 18/19

[§] The chicks inoculated with M101-E17 are the same as those listed in Table IV, but are listed here according to the age at which their CNS was tested. For explanation of other symbols see Table IV.

It is of special interest that on 12 of the 13 chicks inoculated with material from passage M101-E17 tests were done on both, CNS and blood. Four of these (Nos. 8, 10, 11, and 12) were tested simultaneously, *i.e.* 2 days after hatching. The results indicated that the CNS contained probably about 100 times more virus than the blood. The brains and cords of the other chicks, tested 3 to 7 days later, were still more heavily infected than their blood had been on the second day. A pool of serum from chicks No. 521-527, obtained at the same time as the CNS of 5 of this group (Table V), failed to produce paralysis in 24 baby mice even though the CNS was positive in 3 or 4 of 5 tested.

Attempt to infect baby chicks. Only a single attempt was made to determine whether egg-adapted Hawaiian dengue virus could multiply in the CNS of hatched chicks. Seven one-day-old chicks were inoculated intracerebrally with embryo suspension M101-E18. The inoculum contained 10^3 mouse LD_{50}. All 7, as well as 2 un-inoculated chicks of the same age, died 6 or 7 days later. The brains and spinal cords of 3 inoculated chicks were removed immediately after death on the sixth day. Twenty percent suspensions were inoculated into mice. Only one of the 3 suspensions contained traces of virus, not enough to suggest that viral multiplication had taken place.

Discussion. The pronounced predilection of the egg-adapted Hawaiian dengue virus for nervous tissue of chick embryos is of special interest. As far as could be ascertained, Colorado tick fever virus is the only other virus for which similarly preferential growth in the CNS of chick embryos has been reported(6). It is remarkable that this is true for two agents which in their natural host show little if any neurotropic tendencies, while many other viruses which in nature and in experimental mammalian hosts are characteristically neurotropic, are present more or less indiscriminately in various parts of the embryo or in extra-embryonic membranes and fluids. In particular, this applies to the arthropod-borne encephalitis viruses, vesicular stomatitis virus and herpes virus(7). While Gard has reported that the FA strain of Theiler's mouse encephalomyelitis virus could be isolated only from the CNS, but not from other tissues of infected eggs(8), Riordan and Sa-Fleitas found the same virus distributed throughout the egg with the highest concentration in extraneural embryonic tissue(9). The growth of rabies virus in eggs has been studied most recently by Koprowski and Cox(10). According to these authors, the concentration of the virus in the CNS of embryos is only slightly higher than in the extraneural tissues. In contrast to the findings reported above for dengue virus, significant amounts of rabies virus were present in the chorio-allantoic membrane. Yellow fever, in many respects similar to dengue virus, is also less specific than the latter in its distribution in eggs. Fox and Laemmert(2) found that the neurotropic 17D strain attained about the same titer in the CNS and muscle tissue of embryos, and only slightly lower levels in blood, skin, gut and chorio-allantoic membrane.

Despite the preferential invasion of the CNS by dengue virus, no evidence has been obtained that the infection interferes with the normal growth and development of the chicks. The available data indicate that infection does not markedly impede their hatchability, even if eggs are inoculated as early as the eighth day of incubation. In contrast, rabies virus prevents hatching of chicks inoculated before the 15th day of incubation, and the growth and development of embryos inoculated at younger stages is considerably retarded(10). Similarly, the hatchability of embryos infected with 17D yellow fever virus before the 15th day of incubation is almost completely lost, while inoculation on the 17th day fails to diminish it significantly(2). In the case of dengue-infected embryos, despite the relatively high titer of the virus in the CNS tissue, no lesions were found in it and the proliferation of cells seemed to proceed at normal rate, as judged by the large number of mitotic figures seen in the CNS.

Nevertheless, the virus persisted in nervous tissue of chicks until at least ten days after hatching, *i.e.* at least 22 days after inoculation. Comparison of the amounts of virus found in the CNS and in blood of hatched chicks suggest that the early viremia is due to "spill-over" from the CNS. While none of the infected or normal chicks survived long enough to provide material suitable for sero-

---

6. Koprowski, H., and Cox, H. R., *J. Immunol.*, 1947, v57, 255.

7. Beveridge, W. I. B., and Burnet, F. M., *The cultivation of viruses and Rickettsiae in the chick embryo*, London, 1946.

8. Gard, S., *Acta Med. Scand.*, 1943, Suppl. v143.

9. Riordan, J. T., and Sa Fleitas, M. J., *Science*, 1946, v103, 400.

10. Koprowski, H., and Cox, H. R., *J. Immunol.*, 1948, v60, 533.

logical tests, it may be permissible to apply tentatively some of the findings reported for rabies and yellow fever viruses to the dengue problem. Chicks hatching from eggs inoculated with rabies virus between the 15th and 19th day of incubation either died or responded to persisting infection with production of antibody(10). Rabies virus was found to persist in the CNS of one such chick until the 27th day after hatching. Fox and Laemmert(2) did not test the CNS of yellow fever-infected chicks for virus, but found it in blood of several newly hatched chicks. Chicks which had viremia early in life later responded with antibody formation. Further work is required in order to establish whether infection with dengue virus follows a similar pattern.

The failure of hatched chicks to support multiplication of virus after intracerebral inoculation seems to fall in line with the observation that susceptibility of chick embryos decreases with progressing age. In this respect, the Hawaiian strain of dengue virus appears to simulate the 17D strain of yellow fever virus(2).

Summary. 1. The Hawaiian strain of dengue virus has been propagated in chick embryos through more than 90 passages in a series initiated with virus derived from the 101st intracerebral passage in mice. Certain characteristics of the infection of chick embryos have been described in this paper. 2. Susceptibility of chick embryos decreases with increasing age, highest titers being obtained in embryos 4 to 5 days old at the time of inoculation. 3. While sustained propagation of the virus in eggs initially required secondary incubation of more than 5 days, in later egg passages the virus attained maximum titers after 5 days. There was a slight decrease in viral concentration after the seventh day. 4. The infectivity of the virus for chick embryos is of the same order of magnitude as that for mice. 5. Infection with dengue virus does not result in discernible lesions in chick embryos, nor does it appear to affect their growth or their hatchability. 6. The concentration of virus in embryonic brain and spinal cord tissue is 100 to 1000 times higher than in other tissues, with minimal amounts found in extra-embryonic membranes and fluids. 7. Virus has been found to persist in the CNS of chicks until at least ten days after hatching, 22 days after inoculation into the egg. Minimal amounts of virus, much less than present in the CNS, have also been found in the blood of chicks two days after hatching.

Received March 12, 1951. P.S.E.B.M., 1951, v76

Effect of Light on Urinary Coproporphyrin Excretion in Lead-Poisoned Rabbits.* (18642)

R. PIMENTA DE MELLO.† (Introduced by C. J. Watson)

From the Department of Medicine, University of Minnesota Hospital, Minneapolis, Minn.

Certain effects of ordinary light in patients and experimental animals with lead poisoning have been recorded in the literature. Pincussen(1) observed the effect of ultra-violet light (270 mμ), infra-red light and visible light on lead poisoned rats and mice. He concluded that the exposure of these animals to the infra-red and visible light produces an increase of 10-20% in the amount of lead excreted in the urine. Pincussen also mentioned certain "chemical" effects of ultra-violet light in these animals, but did not specify their nature, and a further report has not been found. Rapoport and Rubin(2) studied lead poisoned rats and found that the

---

* Aided by Research Grant No. 345, U. S. Public Health Service.

† Fellow of the Guggenheim Foundation, New York City. (From the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil).

1. Pincussen, L., *Klin. Wchnschr.*, 1933, v12, 275.

2. Rapoport, M., and Rubin, M. I., *Am. J. Dis. Child.*, 1941, v61, 245.

animals kept in the dark showed retardation of growth, but that they appeared "healthy" in that their coats were sleek and they were active. The animals exposed to sunlight were adversely affected in that there were skin and hair changes. They concluded, "the animal experiments further suggest that the photosensitizing action of sunlight on the increased amounts of porphyrin known to be present in persons with plumbism, may contribute in small measure to the seasonal incidence of severe lead poisoning".

The effect of light on urinary porphyrin excretion has also been investigated to a small extent. Thus Hager(3) studied the effect of light of 600-700 mμ on urinary porphyrin excretion. Although there was some indication of irregular increases, they were of questionable statistical significance. The purpose of the present study was to observe the effect of ultra-violet light and ordinary light on urinary coproporphyrin excretion in rabbits with lead poisoning.

Methods. Four adult rabbits of either sex were injected intraperitoneally with a solution of lead acetate (100 mg/kg body wt) in physiological saline solution. R21 received the lead subcutaneously rather than intraperitoneally. Five control rabbits similarly poisoned with lead were kept in the dark by covering the cages with black paper.

Other rabbits were irradiated with unfiltered light from a CH-4 Mercury arc lamp[‡] of maximum ultra-violet intensity at 365 mμ. The hair was shaved from the animal's back and the shaved area was irradiated for one hour at a distance of 12 inches. Additional control rabbits received the same amount of radiation, but did not receive lead.

The concentration of total urinary coproporphyrin was determined by the method of Schwartz and co-workers(4).

Results. The data for urinary coproporphyrin excretion in the rabbits receiving lead and kept either in ordinary light or in the dark are summarized in Fig. 1. Average values for each of the 2 groups are plotted for the 20 days following the injection of lead.

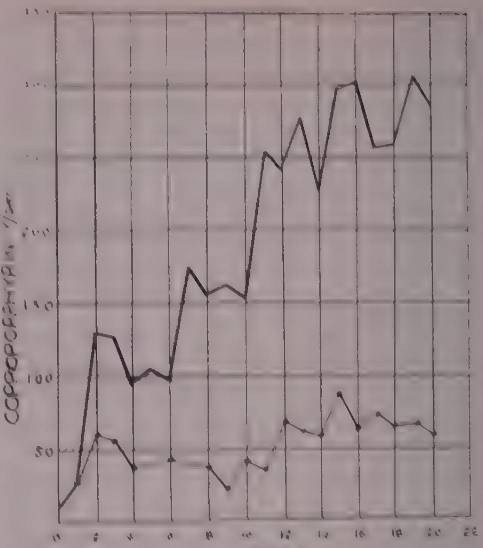


Fig. 1.

Average per diem excretion of urinary coproporphyrin in lead-poisoned rabbits kept either in daylight (upper curve) or in the dark (lower curve).

In the group of lead poisoned rabbits exposed to unfiltered light from a CH-4 Mercury lamp, the urinary coproporphyrin excretion increases by about three fold after the first exposure. The subsequent exposures, however, produce but relatively slight increases. The data are given in Table I.

Three similarly irradiated control rabbits who were not injected with lead showed no increase in urinary coproporphyrin excretion; values ranged from 5-12 μg coproporphyrin per day during the subsequent 6-day period. The average pre-irradiation value was 7.5 μg per day.

Discussion. The present data clearly demonstrate the effect of daylight and of ultra-violet light in materially increasing the urinary coproporphyrin in rabbits with acute lead poisoning. The reason for this increase is not clear, but two possibilities are considered: (1) that the light in some way mobilizes excessive porphyrin already formed and present in the tissues, as a result of the lead poison-

---

3. Hager, B. G., *Klin. Wchnschr.*, 1939, v18, 1045.

[‡] Black Light Products, Inc., Chicago, Ill., Model No. BL-2.

4. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.

TABLE I. Effect of Irradiation with Unfiltered Light from a CH-4 Mercury Lamp on Excretion of Urinary Coproporphyrin in Lead-Poisoned Rabbits. Urinary coproporphyrin in μg/24 hr.

Day	R18	R21	R23
10		250	210
11		275*	220*
12		950	550
13			520
14		480	480
15	110	450*	350*
16	150*	650	655
17	650	550	400
18	330	470	380
19	180	270	360
20	250	270	250
21	110	273	280*
22	160	150	360
23	80	80	200
24	110	82*	
25	180	200	
26	170*	90	
27	350	88	
28	390	88	
29		60	
30	150	60	
31	150*	50	
32	180	50	
33	210	50	
34	110		

* UV light exposure on day indicated. Lead given on day 0 in each instance. No analyses were made on days 1-14 on rabbit R18 and days 1-10 on rabbits R21 and 23.

ing, (2) that the light stimulates or brings about a rapid increase in coproporphyrin formation. The observation (Table I) of diminishing effect of repeated ultra-violet exposures, is compatible with either possibility, as in the first instance it could be regarded as exhaustion of stored porphyrin, and in the second, of the cellular potentiality to make the excessive porphyrin. In a recent study of the urinary coproporphyrin in lead poisoned rabbits, carried out with the aid of glycine containing N^{15}(5) the findings indicated that the increased porphyrin was more likely formed as a result of the lead, rather than a heightened excretion of preformed porphyrin. In the present study, however, it is entirely conceivable that the effect of light is to increase the excretion of preformed porphyrin.

Summary and conclusions. 1. Light exposure increases the urinary coproporphyrin excretion in rabbits with lead poisoning. 2. The increase is especially marked after ultraviolet radiation. Subsequent radiation, however, fails to cause a renewed rise or is followed by only a small increase. 3. Whether the increase following light is due to mobilization of preformed porphyrin, or to increased porphyrin formation, has not been determined.

I am deeply indebted to Dr. C. J. Watson and Dr Samuel Schwartz for many valuable suggestions, and interest in this work.

---

5. Grinstein, M., Wikoff, H. N., Pimenta de Mello, R., and Watson, C. J., *J. Biol. Chem.*, 1950, v182, 723.

Received September 25, 1950. P.S.E.B.M., 1951, v76

Sulfhydryl Inhibition as a Mechanism in the Effects of ACTH and Cortisone.* (18643)

GEORGE E. ANDERSON, LEON L. WIESEL, ROBERT W. HILLMAN, AND WILLIAM M. STUMPE. (Introduced by S. Seifter)

From Department of Medicine, Brooklyn Hospital, Brooklyn, N. Y.

Suppression or partial inhibition of cellular enzyme systems seems to be at least one widespread mechanism of action of the C_{11}-oxygenated adrenal-cortical steroids. The demonstrated disrupting effect(1) of these steroids on phases of carbohydrate metabolism may well be mediated thru inhibition of enzyme systems essential to the normal processing of glucose by the organism. If such inhibitory effect is as widespread as is suggested, it would seem reasonable to anticipate the presence of some common denominator of mechanism in the systems affected. It has been demonstrated that ACTH reduces availability of sulfhydryls(2) to the organism, a deprivation which in obligate sulfhydryl enzyme systems could effectively block enzymatic activity(3). Carboxylase, phosphoglyceraldehyde oxidase, phosphoglucomutase, pyruvate oxidase, adenosinetriphosphatase are all enzyme systems allegedly dependent on SH groups for their activity(3). Those enzyme systems of the tricarboxylic acid cycle which depend on Coenzyme A for their functional integrity(4) all require sulfhydryls, since Coenzyme A is itself a sulfhydryl obligate. Besides the C_{11}-oxygenated adrenal-cortical steroids, other agencies are known to rob the metabolic pool of its sulfhydryl groups by the oxidation of –SH to SS. Notable among these is ascorbic acid(5) thru its oxidation product, dehydroascorbic acid, which oxidizes SH to SS.

---

* Aided by a grant from the Mezger Pharmacal Co. Ascorbic acid supplied by Hoffmann-La Roche.

1. Conn, J. W., *J. Lab. and Clin. Med.*, 1948, v33, 651; Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 441; Conn, J. W., Louis, L. H., Johnston, M. W., *Science*, 1949, v109, 279.

It seemed feasible first to study in parallelism the effects of cortisone and of massive administration of ascorbic acid on the sulfhydryl content of human whole-blood.

To establish base-lines and at the same time to test methodology, whole-blood levels of sulfhydryls and of ascorbic acid were determined in 20 normal individuals, using the method of Woodward and Fry(6) for –SH and the indophenol method of Jung for ascorbic acid(7) making the necessary quantitative –SH corrections for the reducing effects of the ascorbic acid present. The results of these determinations were found to fall well within the range of expectancy. Massive doses of ascorbic acid (up to 2 g by vein) were then rapidly administered to 8 of these normal individuals, repeating the same blood determinations before and after the ascorbic acid administration. The same procedure was then carried out in 5 patients with typical rheumatoid arthritis who had previously shown on one or more occasions temporarily striking but evanescent clinical improvement on massive doses of ascorbic acid administered by vein (without DOCA). In addition, whole-blood sulfhydryl determinations were made in 5 other rheumatoid arthritics before and after completely effective therapy with cortisone.

Findings. The "before and after" –SH readings on the whole blood of all 3 groups were too variable and small in their differences to be experimentally significant. The negative findings on humoral (blood) assay for sulfhydryls after administration of known –SH depletors suggested that the blood might in no wise reflect a dearth or sufficiency of these groups in the cells themselves, where, after all, the intracellular enzyme systems would be functioning and where, accordingly,

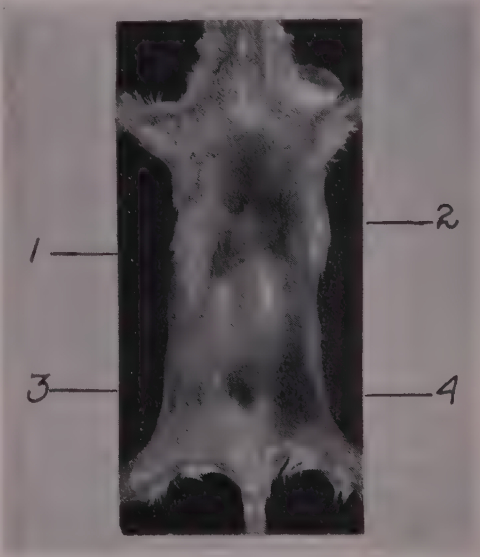


FIG. 1.
1—Saline-India ink control.
2—Saline-hyaluronidase-India ink control.
3—Hyaluronidase after cortisone.
4—Hyaluronidase after G-SH.

---

2. Conn, J. W., *Proc. Am. Diab. Assn.*, 1948, v8, 213.

3. Barron, E. S. G., *Biol. Sympos.*, 1943, v10, 27; Barron, E. S. G., Singer, T., *Science*, 1943, v97, 356; Houssay, B. A., *Am. J. Med. Sci.*, 1950, v219, 353.

4. Kaplan, N. O., Lipman, F., *J. Biol. Chem.*, 1948, v174, 37; Snell, E. E., Brown, G. M., Peters, V. J., Craig, J. A., *et al.*, *J. Am. Chem. Soc.*, 1950, v72, 5349.

5. Hopkins, F. G., Morgan E. S., Lutwak-Mann, C., *J. Biochem.*, 1938, v32, 1829.

6. Woodward, G. E., and Fry, E. G., *J. Biochem.*, 1932, v97, 465.

7. Jung, A., *Schweiz. Med. Wochenschr.*, 1934, 583.

conversions from –SH to SS would be registered by failure of these systems. To shed further light on sulfhydryl relation to enzyme function in closer proximity to fixed tissue cells, the skin-spreading activity of the hyaluronidase enzyme system was explored. This system was chosen since it had been shown by Opsahl(8) and by Seifter(9) to be at least partially inhibited by adrenal cortical hormone *in vivo* as well as *in vitro* and since the spreading effect of hyaluronidase in skin would lend itself to rough quantitative visual appraisal of activity and reversibility of such activity.

Procedure. Depilated skin of white mice was used as a proving ground for the influence of free sulfhydryls (reduced glutathione) in reversing the known inhibitory effect of cortisone on the skin-spreading phenomena of mixtures of hyaluronidase in India ink, using the skin technic of Opsahl(8). A group of 18 white mice averaging 15 to 20 g in weight were prepared by depilation of the skin of the abdomen and were kept on a high-protein diet for 3 days to insure against primary depletion of –SH groups. As a control 0.1 cc of normal saline marked with India ink was injected intracutaneously in the right upper quadrant. As a further control, 0.1 cc of saline containing 75 turbidity units of commercial hyaluronidase per cc and marked with India ink was injected intracutaneously into the left upper quadrant. The mice were then prepared further by the subcutaneous injection of 1 mg of cortisone acetate given twice at twelve-hour intervals. Six hours after the second dose, 0.1 cc of normal saline containing 75 turbidity units of hyaluronidase per cc marked with India ink was injected into the skin of the right lower quadrant. After an interval of one-half hour each animal was given 40 mg of reduced glutathione in saline solution intraperitoneally. One-half hour later, 0.1 cc of hyaluronidase in saline was injected into the left lower quadrant intradermally. The spread of the India ink in the skin was measured in 15 minutes and again after 24 hours.

RESULTS

		Diameter of spreads		
Skin tests	Total	0.5 cm	1.0 + cm	1.5 + cm
1. Saline control	18	12	6	
2. Hyal. control	18		13	5
3. Hyal. after cortisone	18	13	5	
4. Hyal. after G-SH	18		8	10

Negative findings in 3 animals (or Spreads 2, 3, and 4 are equal).

In 15 of the 18 mice, the original or an increased skin-spread was re-established, presumably thru the de-inhibiting effect of reduced glutathione in cortisone-inhibited animals.

In a control group of mice the procedure was repeated, substituting oxidized glutathione (G-SS-G) for G-SH. There was no appreciable change in the cortisone-inhibited hyaluronidase spreading phenomenon in these animals.

Conclusion. Evidence has been presented to suggest that ACTH and cortisone produce their inhibitory action on the skin-spreading effect of the hyaluronidase enzyme system thru the agency of sulfhydryl deprivation. This finding warrants further exploration of enzyme systems known to be inhibited or depressed by the C_{11}-oxygenated adrenal-cortical steroids for sulfhydryl deficiency created by these steroids as a common denominator in the inhibitory effect. The humoral (whole-blood) content of –SH groups after clinically effective therapy with cortisone does not necessarily reflect a sufficiency of this factor in the fixed tissues where its influence on the intracellular enzyme systems would be registered by the functional integrity of these systems.

Received March 12, 1951. P.S.E.B.M., 1951, v76.

---

8. Opsahl, J. C., White, A., Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, 1950, v52, 106.

9. Seifter, J., Baeder, D. H., Dervenis, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 136.

The Xanthine Oxidase Factor from Liver.* (18644)

Jean P. Kring and J. N. Williams, Jr. (Introduced by C. A. Elvehjem)

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Westerfeld and Richert(1) observed that in weanling rats fed a purified 21% casein diet the initial low level of liver xanthine oxidase remained essentially unchanged for at least 6 weeks. Feeding a 21% crude casein diet produced a significant increase of liver xanthine oxidase after 4 weeks, but not within 2 weeks. Addition of 10% Wilson's whole liver or other natural unpurified foodstuffs to the purified rations increased the xanthine oxidase activity to almost normal levels within 2 weeks. They considered that some unidentified factor present in certain natural materials must be supplied in the diet of the weanling rat for production of normal levels of liver xanthine oxidase. Williams and Elvehjem(2) have shown that liver xanthine oxidase in the adult rat is a sensitive index of amino acid availability in dietary proteins. They demonstrated that methionine in dietary casein is not readily available to the rat but that if either acid hydrolyzed casein or a mixture of amino acids simulating casein is fed as a source of protein, the rat appears to utilize the amino acids much more completely. They also observed that the effects of the low availability of methionine in casein upon liver xanthine oxidase could be overcome by increasing the dietary level of casein to 40 per cent. In addition, Williams *et al.*(3) have shown that the liver xanthine oxidase activity of young rats is especially sensitive to a methionine deficiency.

The studies presented in this paper were carried out to investigate whether an increase in liver xanthine oxidase activity with natural diets over purified diets is due to an unidentified factor or to a better biological value of protein when the natural foodstuffs are incorporated into the diet. Schaefer *et al.*(4) have observed that both young and adult mink fed highly purified rations supplemented with all the known crystalline vitamins require additional unidentified factors for normal nutrition. Preliminary evidence from the fractionation of the factors in fresh raw liver indicated that methanol extraction removed 1 or more of the factors and that the insoluble residue contained still other factors. It appeared possible that these factors might be related to the unidentified xanthine oxidase factor suggested by Westerfeld and Richert (1).

In preliminary experiments in which defatted whole liver (Viobin) was substituted for part of the protein in a purified casein ration, the liver xanthine oxidase activity of young rats did not respond to any greater extent than with the basal casein ration. Therefore, it was decided to test the ether-soluble fraction of whole liver to determine if any factor might have been removed during the commercial defatting process. Since dextrin is known to favor bacterial synthesis of various dietary factors in the intestinal tract, it appeared important to observe the effect of a dextrin ration upon xanthine oxidase activity of young rats. Another dextrin ration containing sulfasuxidine was also fed to eliminate the effects of bacterial synthesis and therefore to observe the effects of the dextrin *per se*.

Experimental. Weanling, male, albino rats of the Sprague-Dawley strain were employed as experimental animals. The basal ration consisted of 18 g casein, 5 g corn oil, 4 g Salts

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and from the Nutrition Foundation, Inc., New York.

1. Westerfeld, W. W., and Richert, D. A., *J. Biol. Chem.*, 1950, v184, 163.

2. Williams, Jr., J. N., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v181, 559.

3. Williams, Jr., J. N., Denton, A. E., Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1949, v72, 386.

4. Schaefer, A. E., Tove, S. B., Whitehair, C. K., and Elvehjem, C. A., *J. Nutrition*, 1948, v35, 157.

TABLE I. Effects of Various Liver Fractions and Dextrin Upon Response of Liver Xanthine Oxidase Activity in Weanling Rats.

Group	No. of rats	Carbohydrate	Supplement	Xanthine oxidase activity after 4 wk (μl O_2/hr/g rat liver)
I	11	Sucrose	0	89 ± 7*
II	7	”	Methanol extr. liver	85 ± 0.3
III	7	”	Liver residue after methanol extr.	116 ± 2
IV	7	”	Methanol extr. liver + residue	102 ± 8
V	7	”	Viobin defatted liver	90 ± 3
VI	7	”	Ether extr. liver	89 ± 7
VII	7	”	Liver residue after ether extr.	127 ± 7
VIII	7	”	Ether extr. liver + residue	117 ± 11
IX	7	”	Whole liver	144 ± 5
X	9	Dextrin	0	142 ± 19
XI	9	”	1.5% sulfasuxidine	136 ± 25

* Standard error of the mean.

IV(5), 2 g vitamin mix(2), and carbohydrate (sucrose or dextrin) to make 100 g. In addition the animals were given 2 drops of haliver oil orally per week. The weanling rats were separated at random into 11 groups and fed the rations shown in Table I. Total nitrogen of the liver fractions and residues was determined by a semi-micro Kjeldahl procedure (6). The liver residues were added to the ration at a 10% level, and the methanol and ether soluble fractions were added at a level equivalent to 10% whole liver. In all cases the liver fractions and residues were added to the basal ration at the expense of casein on a nitrogen basis, making a total of 18% protein in the diet (nitrogen x 6.25 = protein). The methanol extract of liver and the residue were prepared as described by Schaefer *et al.* (7). The ether soluble liver fraction was prepared by grinding fresh beef liver, mixing with an equal volume of distilled water in a Waring Blendor, drying in a hot air oven at 70°C and grinding to a powder in a Wiley Mill. The powder was refluxed with ether in a macro Soxhlet apparatus until the ether was no longer colored. Most of the ether was then removed by distillation. After 4 weeks on the respective diets, the animals were sacrificed and liver xanthine oxidase activity determined at 30°C by the method of Axelrod and Elvehjem(8).

Results and discussion. The results of the xanthine oxidase determination are presented in Table I.

It appears that the methanol extract contained no factor which increased the enzyme activity over that obtained on a sucrose basal ration. However, an increase in activity with the residue was noted indicating that if a factor is involved it is probably contained in the liver residue fraction. Upon combination of the methanol extract with the insoluble residue, a significant and at present unexplainable decrease in the enzyme activity was observed.

With Viobin defatted liver no increase in activity over that of the sucrose basal ration was observed, as mentioned above. It appeared possible therefore that a fat-soluble factor had been removed during the defatting process. However, with the ether extract no increase in activity over that given by the sucrose basal ration was observed. The ether extracted residue gave an increase similar to that obtained with the methanol extracted residue. Again a slight decrease in activity was noted here as with the combined methanol fractions when the ether extract and ether-insoluble residue were fed together.

If the response of xanthine oxidase to the feeding of the liver residues were due to an unidentified factor retained in the residue, it

---

5. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

6. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, 1943, v37, 354.

7. Schaefer, A. E., Whitehair, C. K., and Elvehjem, C. A., *J. Nutrition,* 1947, v35, 147.

8. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, v140, 725.

appears that essentially the same results should be obtained with the liver residues after extraction as with whole liver. However, as shown in Table I, whole liver causes a significantly greater response than either of the 2 liver residues fed. It is possible that extraction with organic solvents may have decreased the biological quality of the liver protein or the biological activity of the unidentified factor.

The increased activity of the group receiving the whole liver might have resulted from the ingestion of proteins in which the amino acids were more effectively utilized than in the purified casein. As already shown, methionine appears important in xanthine oxidase activity (2,3). Amino acid analyses(9) of liver and casein show that both contain almost identical amounts of methionine, but that the cystine concentration in liver is approximately 1.4 ± 0.1% while in casein it is present at a level of 0.36 ± 0.04%. Hence it is possible that the cystine could spare enough methionine to result in a higher level of enzyme activity when the liver was incorporated into the diet.

An increase over the activity of the sucrose basal ration was observed with both diets containing dextrin whether sulfasuxidine was included or not. This leads us to believe that the effect of the dextrin was not through bacterial synthesis of a xanthine oxidase factor but rather by increasing utilization of the ingested ration. Since dextrin is probably less easily digested and absorbed than sucrose because of the size of the molecule, the ration would be held in the intestinal tract longer, thus allowing more complete digestion and utilization of the diet.

Records of the food consumption and weight gains for all groups of animals were kept, but no correlation between these factors with the differences in enzyme activity was observed.

It is possible that extraction of liver with solvents other than those reported might give positive results. It is our interpretation however, that the increase in enzyme activity noted with the liver residue is probably better explained by the superior biological value of the liver proteins as compared to purified casein.

Summary. 1. The xanthine oxidase factor of dietary liver has been investigated by feeding various liver fractions and residues to weanling rats and observing the response of the rat liver xanthine oxidase activity to the various fractions. 2. The results indicate that the unidentified factor is not extractable by methanol or ether but that it is retained in the extracted liver residue. Whole untreated liver gave a more significant response than any fraction or residue tested. 3. Dextrin in the place of sucrose in the diet with no liver or liver fraction added gave a response as great as that given by whole liver. 4. It appears that the increase in xanthine oxidase activity observed in the weanling rats fed liver or liver residue is possibly due to better biological value of the liver protein rather than to a trace factor in the liver.

The authors wish to thank Mr. R. J. Lalor and Mr. W. L. Leoschke of this department for furnishing us with samples of the methanol soluble liver fraction and the liver residue after methanol extraction.

Received March 12, 1951. P.S.E.B.M., 1951, v76.

---

9. Block, R. J., and Bolling, D., *The amino acid composition of proteins and foods,* Springfield, 1945, 303.

Observations on the Cystic Stage of *E. histolytica* in Abscesses of the Human Bowel. (18645)

Ryle A. Radke. (Introduced by O. Felsenfeld)
From the U. S. Army Hospital, Fort Knox, Ky.

The exact place and formation of cysts of *E. histolytica* in human infections has not been decided. Most text books venture the opinion that the cystic stage is formed in the fecal stream and not in the bowel wall, where this ameba species causes abscess formations followed by ulcerations. Warthin(1) described the presence of cysts and pre-cysts of *E. histolytica* in amebic lesions of the testis and of the epididymis. No other references to such observations were found by this worker in the medical literature. Using a technic consisting of careful cleansing of the fecal stream overlying amebic abscesses, by means of aspiration employing a woven catheter and ear, nose and throat suction machine, followed by additional cleansing by aspiration with 1 cc bacteriologic pipette, the writer aspirated material containing cysts of *E. histolytica* from abscesses in the bowel wall of 25 patients with intestinal amebiasis.

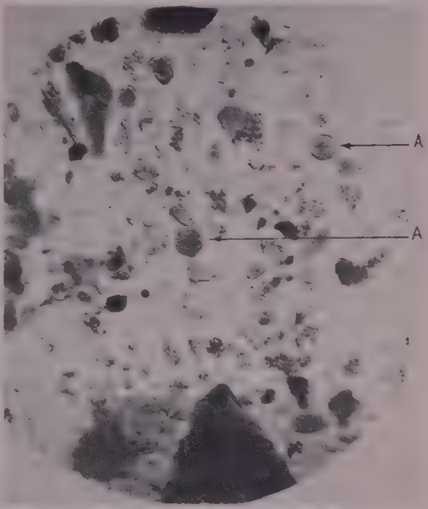


Fig. 1.
Cysts of *Endamoeba histolytica* aspirated from miliary abscess of the human bowel. (×100). A. Cysts of *Endamoeba histolytica*.

Fig. 1 represents cysts of *E. histolytica* pipetted from a minute rectal abscess in a 26-year-old white male with chronic amebiasis. This material was stained with modified iodine stain and photographed in a wet preparation. These findings suggest that the cysts of *E. histolytica* may be formed within the tissues rather than in the fecal stream.

---

1. Warthin, A. S., *J. Infect., Dis.*, 1922, v30, 559.

Received March 19, 1951. P.S.E.B.M., 1951, v76.

Blood Volume of the Normal Female as Determined with P32 Labeled Red Blood Cells.* (18646)

Nathaniel I. Berlin, Grace M. Hyde, Robert J. Parsons, John H. Lawrence, and Shirley Port.
From the Donner Laboratory of Medical Physics, University of California, Berkeley, and the Pathology Laboratory, Highland-Alameda County Hospital, Oakland, Calif.

During the course of an investigation of the changes in the blood volume in pregnancy it became apparent that a re-evaluation of the blood volume standards in normal women was necessary.

As measured with the dye T-1824 in normal males and females, the blood volume and plasma volume were significantly lower in the female(1,2), and with the red dye methods there was no marked difference between males and females(3,4). Only 2 blood volumes were reported in normal females using the

---

* This study was supported in part by the Atomic Energy Commission.

washed P32 labeled red blood cells(5) and none using radioactive iron. Reeve and Veall (6) did not state the sex of their normal subjects. The present study shows that the blood volume per kilogram of body weight of the normal female is slightly lower than the normal male due principally to a lower total red cell volume and due to the lower average density of the female(7).

Methods. The blood volume was determined in 16 healthy females on the nursing and office staff of the hospital with P32 labeled red cells using a modification of the Hevesy and Zerahn method(8,9). The average age of these subjects was 31.3 years and the range was 22 to 48 years.

Results. The blood volumes are presented in Table I.

TABLE I. Normal Females.

Subject	Body wt, kg	Blood vol.*	Total red cell vol.*	Plasma vol.*	Hematocrit
1	52.7	63.4	27.6	35.5	43.5
2	66.8	57.5	24.1	33.4	42
3	65.9	49.6	22.1	27.3	44.5
4	49.5	65.4	24.2	41.2	37
5	57.8	60.5	25.4	35.1	42
6	81.4	66.2	25.2	40.4	38
7	54.5	51.2	21.1	29.9	41
8	44.5	77.5	30.9	46.6	40
9	61.4	71.1	32.7	37.7	46
10	58.6	63.3	26.6	36.1	42
11	59.1	64.5	27.7	36.1	43
12	57.3	65.6	28.8	36.1	44
13	56.8	74.3	31.9	41.7	43
14	62.7	61.1	26.9	33.6	44
15	50.5	64.9	25.9	38.3	40
16	51.8	74.9	31.5	43.4	42
Avg		64.4	27.0	37.0	42
		±1.9†	±0.84†	±1.19†	±0.61†

* In cc/kg body wt.
† Stand. dev. of the mean.

Discussion. The average blood volume of the normal female is 64.4 cc/kg as compared with 68.6 cc/kg body weight in the male. The average total red cell volume is 27.0 cc/kg in the female as compared with 29.9 cc/kg in the male(9). The difference in the plasma volume was not significant; the differences in average total blood volume and average total red cell volume are statistically significant.

A part of this decrease in total red cell volume may be related to the fact that the index used is cc/kg body weight. Studies of total body water show that in women the percent of the body which is water is less than in males; this is due to the greater percentage of adipose tissue which contains less water than the other tissues(7). It is probable that calculated on the basis of "fat free" body weight, the total red cell volume of the female would be in better agreement with that of the male. In debilitating disease states the total red cell volume of the female probably should be compared to the normal male because of the loss of body fat in these conditions.

These studies show that blood volume difference between males and females is not as great as has been reported with Evans blue dye method, and is in good agreement with the values reported with the red dye method.

Conclusion. As measured in 16 normal females with P32 labeled red blood cells, the blood volume was 64.4 cc/kg; total red cell volume was 27.0 cc/kg with the plasma volume 37.0 cc/kg body weight. These values for the total red cell volume do not differ markedly from those observed in a similar group of males when the difference in total body fat in the two sexes is taken into consideration.

---

1. Gibson, T. G. and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, v16, 301.

2. Hallock, P., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 11.

3. Rowntree, L. G., Brown, G. E., and Roth, G. M., *The volume of the blood and plasma in health and disease*, W. B. Saunders Co., Philadelphia, 1929.

4. Haden, R. L., *Am. J. Med. Sc.*, 1938, v196, 493.

5. Mayerson, H. S., Lyons, C., Parsons, W., Nieset, R. T., and Trautman, W. V., Jr., *Am. J. Physiol.*, 1948, v155, 237.

6. Reeve, E. B., and Veall, N. B., *J. Physiol.*, 1949, v108, 12.

7. Steele, J. M., Berger, E. Y., Dunning, M. F., and Brodie, B. B., *Am. J. Physiol.*, 1950, v162, 313.

8. Hevesy, G. and Zerahn, K., *Acta. Physiol. Scand.*, 1942, v4, 376.

9. Berlin, N. I., Lawrence, J. H., and Gartland, J., *J. Lab. and Clin. Med.*, 1950, v36, 435.

Received April 2, 1951. P.S.E.B.M., 1951, v76.

Parabiosis Intoxication. (18647)

John C. Finerty and Theodore C. Panos.

From the Departments of Anatomy and Pediatrics, University of Texas, Medical Branch, Galveston.

A condition which is peculiar to the parabiotic state interferes with survival of some pairs longer than about 2 weeks. Its etiology has never been satisfactorily explained. It has been described principally in the German literature and has been called "Parabiose Vergiftung", parabiosis poisoning, parabiosis intoxication, parabiotic disease, parabiotic disharmony, and heterogeneous parabiosis. It was probably originally described by Ranzi and Ehrlich(1), and first mentioned in American literature when Dragstedt and Cooper(2) stated: "It shall be noted that many pairs die suddenly from some cause at present unknown. There are no definite post-mortem abnormalities. In other cases, particularly when there is marked discrepancy in size or strength between animals at operation, the smaller may fail to grow, become weak and anemic and die in the course of 3 to 4 weeks. Stunting in one of a pair of parabiotic twins has been occasionally seen, but is unaccounted for." There are many differences of opinion regarding the incidence, etiology and inevitability of parabiosis intoxication. Some authors believe it is an inevitable consequence of the parabiotic state, others that it is a result of constitutional incompatibility between individual unrelated animals whereas others believe it to be a result of faulty operational technic. Most experimental evidence supports the concept of incompatibility which expresses itself as an anaphylactoid reaction(3). Mayeda(4) attributed "heterogeneous parabiosis" to blood incompatibility between the animals, resulting in hemolysis in one partner. In his experience, 67% of all pairs showed symptoms of intoxication, with a somewhat greater incidence if the partners were not littermates. Herrmansdorfer(5) emphasized cachexia as the primary symptom of parabiosis poisoning, induced by disturbance in appetite of the smaller "parasitic" partner and changes in blood volume. He found 60-70% disharmony, and stated that the condition might be more or less pronounced, so that those animals with greater power of resistance could recover. According to Nissen(6), parabiosis intoxication is essentially a "protein poisoning" and his findings of increased plasma cells and depleted adrenals were attributed to the defensive reaction against protein breakdown products. He compared his finding of hyperplasia of the thymus and lymphoid tissue in the larger partner with Status Thymo-lymphaticus in humans, and suggested that the latter may be the result of derangement in the parabiotic existence of mother and fetus during pregnancy. It now seems probable that Nissen was comparing the essentially normal lymphoid tissue of the larger partner with the extreme atrophy of the smaller intoxicated parabiont. Nevertheless, it may be profitable to consider the possibility that incompatibility between two rats in parabiosis may be an experimental counterpart of incompatibility between mother and fetus. For example, in erythroblastosis fetalis the fetus is the intoxicated partner, and it is conceivable that in the toxemias of pregnancy the mother is the victim. Other interpretations of the etiology of intoxication have been: protein poisoning as a result of humoral exchange of toxins(7,8); chronic progressive anaphylaxis(9,10); nutritional

---

1. Ranzi, E. and Ehrlich, H., *Z. f. Immunitatsf. u. exp. Ther.*, 1909, v3, 38.

2. Dragstedt, L. R., and Cooper, E. F., *Am. J. Physiol.*, 1923, v67, 48.

3. Møller-Christensen, E., *Acta Path. et Microbiol. Scand.*, 1932, v9, 55.

4. Mayeda, T., *Deutsche Z. f. Chir.*, 1921, v167, 295.

5. Herrmansdorfer, A., *Deutsche Z. f. Chir.*, 1923, v178, 289.

6. Nissen, R., *Z. f. d. ges. exper. Med.*, 1923, v35, 251.

7. Sauerbruch, F., *Munchen med. Wchnschr.*, 1923, v70, 866.

8. Ernst, M., *Deutsche Z. f. Chir.*, 1929, v221, 74.

9. Schmidt, G., *Deutsche Z. f. Chir.*, 1922, v171, 141.

TABLE I. Incidence of Parabiosis Intoxication and Effects of Attempted Therapy in 100 Pairs of Parabiotic Rats.

	No. of pairs	Died (%)	Recovered (%)	Asymptomatic (%)
Non-littermate pairs				
Untreated	40	26 (65)	10 (25)	4 (10)
Antihistaminic-treated	29	18 (62)	9 (31)	2 (7)
Cortisone-treated	9	6 (67)	3 (33)	
Littermate pairs	10	5 (50)		5 (50)
Surgical deaths	12			

differences(11); Bartonella anemia(12); and poor operative technic(13). Since the advent of antihistamine drugs, the possibility of their usefulness in combating parabiosis intoxication has been considered. Simonsen and Christensen(14) reported failure in their attempt to elucidate this problem, but it was probably not a fair test since their experimental approach was so rigorous. They united mice with rats and were unable to prolong the existence of the pair by antihistaminic treatment.

The purpose of the present work is to describe the incidence and symptoms of intoxication in 100 pairs of male rats, and to report the results of attempts to modify the condition by treatment with an antihistaminic and with cortisone.

Experimental procedure. Male rats obtained from the Holtzman-Rolfsmeyer Co. were put in parabiosis at the age of 31 days, according to the method of Bunster and Meyer (15) except that metal skin clips were used instead of sutures. Each pair was examined carefully twice daily for the succeeding 7 weeks. The distribution of these pairs into experimental groups is shown in Table I. The pairs which were treated with antihistaminic therapy were injected twice daily intraperitoneally with 1.0 mg chlorocyclizine hydrochloride (Perazil, Burroughs Wellcome and Co.) from the 3rd to the 21st day following operation. Those which were treated with cortisone received 0.75 mg cortisone acetate (Cortone acetate, Merck and Co.) from the 3rd to the 21st day.

Results and discussion. The first appearance of parabiosis intoxication was hyperemia (redness and dilation of blood vessels of ears, feet, tail and external genitalia), of one partner, and/or slight size difference. During the next few days a progressive increase in size difference occurred with the hyperemic partner remaining larger and the smaller partner becoming increasingly pale and anemic in appearance. About a day before death the smaller, anemic partner usually became sluggish, moribund, and in our experience always died first. Rarely pairs were observed in which both rats were hyperemic, or both anemic, a condition which persisted for several days followed by recovery or by alteration of one partner so that the usual symptoms obtained. If the larger rat is separated soon after the death of its partner, it usually survives in good health.

In this series, initial signs of intoxication appeared between the 8th and 14th days after pairing, followed by death within 2 to 8 days or by recovery. Onset of signs was never observed after the 21st post-operative day, and few deaths occurred thereafter.

Autopsy of pairs in which one partner had died revealed that the larger surviving partner was essentially normal, except for slightly enlarged adrenal glands. The smaller, intoxicated animal exhibited extreme hypertrophy of adrenals, extreme atrophy of the thymus and a completely empty gastro-intestinal tract. Adhesions or infections were found in some pairs which were intoxicated, but not

---

10. Nickau, B. and Duschl, L., *Deutsche Z. f. Chir.*, 1925, v191, 221.

11. Kojima, K., *Deutsche Z. f. Chir.*, 1931, v232, 578.

12. Sauerbruch, F. and Knake, E., *Klin. Wchnschr.*, 1936, v15, 884.

13. Pearlman, L. R. and Kolpakow, I. V., *Vrach. Delo.*, 1938, v20, 871.

14. Simonsen, M. and Christensen, R., *Acta Path. et Microbiol. Scand.*, 1950, v27, 323.

15. Bunster, E. and Meyer, R. K., *Anat. Rec.*, 1933, v57, 339.

in the majority. Hemoglobin determinations indicated that homogeneous pairs had comparable hemoglobin concentrations in their blood, but in pairs with a marked "hyperemia-anemia" difference the anemic parabiont had only one-third to one-half as much hemoglobin. Hyperhemoglobinemia and polycythemia have been observed in the "hyperemic" partner of intoxicated pairs by Diepenhorst and de Vaal(16). They also have been unable to correlate hematological phenomena induced by parabiosis with any known blood group differences. The color difference has been attributed to differences in blood pressure between the partners which results in one rat "bleeding into the other", but blood pressure determinations in heterogeneous pairs show no constant pattern which would support such a contention(17).

The incidence and results of attempts at treatment of parabiosis intoxication are shown in Table I. Of the 100 pairs, 12 died during the operation or within a few days from the effects of surgery (death from anesthesia, hernia and fighting between partners). It can be seen that no significant difference exists in the incidence of intoxication between those pairs which were untreated, antihistamine-treated or cortisone-treated. Also no change was noted in the duration or severity of the condition as a result of therapy. Littermate pairs showed only slight difference in percentage of death from intoxication, but marked difference in this group is shown by the fact that half of them showed no signs of intoxication at all.

Changes in the adrenals and thymus as a result of parabiosis intoxication simulate the responses of an animal to severe stress and probably are comparable to Selye's "adaptation" phenomena. It may be of importance to consider this factor in evaluating results of endocrine studies in which the parabiosis technic is used(16).

From our data and from a survey of the literature, we conclude that parabiosis intoxication is a result of constitutional incompatibility between the partners, complicated by infection, inanition and other factors which either accentuate or mask the primary signs. In spite of the belief of Pearlman and Kolpakow(13) that it can be explained on the basis of poor operative technic, the facts that littermates show less intoxication and that use of highly inbred strains practically eliminates the condition, as shown in the work of Braun-Menendez(18), and Huff, Trautman and Van Dyke(19), are cogent arguments for a genetic basis. Antihistaminic and cortisone treatment are ineffective in altering the course or incidence of the intoxication.

Summary. Mortality of rats from parabiosis intoxication in non-littermate pairs was 64%, signs of intoxication followed by recovery were shown by 28% and 8% remained asymptomatic. Treatment of non-littermate pairs with an antihistaminic or cortisone had no effect on the incidence or severity of the intoxication. Littermate pairs showed less tendency toward intoxication, but those which were affected died as readily as non-littermates.

---

16. Diepenhorst, M. J., and de Vaal, O. M., *Acta Physiol. et Pharmacol. Neerl.*, 1950, v1, 342.

17. Hall, C. E., and Hall, O., personal communication.

18. Braun-Menendez, E., personal communication.

19. Huff, R. L., Trautman, R. and Van Dyke, D. C., *Am. J. Physiol.*, 1950, v161, 56.

Received April 2, 1951. P.S.E.B.M., 1951, v76.

Reduction of Antidiuretic and Pressor Activity Associated with Adrenocorticotropic Hormone (ACTH) Preparations. (18648)

W. O. Reinhardt and Choh Hao Li.*†

From the Division of Anatomy and Department of Biochemistry, University of California, Berkeley, Calif.

The preparation of purified ACTH from whole pig or sheep pituitary glands, or from dissected beef anterior lobe material, may involve contamination of the final product by substances having antidiuretic and pressor activity similar to that found in posterior lobe extracts. Since clinical and experimental use of ACTH preparations requires relative freedom from posterior lobe contaminants in order to avoid undesirable side effects of these substances, it is thought to be of value to present a method which reduces to markedly low values the antidiuretic and pressor activity found in various preparations of sheep ACTH.

Materials and methods. Four types of ACTH preparations have been tested: (A) ACTH protein prepared according to the method of Li, Evans and Simpson(1); (B) ACTH peptide mixture prepared by pepsin digestion of ACTH protein (A) according to Li(2); (C) ACTH protein prepared according to a method to be reported by Li and Reinhardt(3); and (D) ACTH acid peptides prepared from ACTH protein preparation (C), according to a method described by Li(4), in which the acid hydrolysate is freed from the unhydrolyzed protein and isolated. These various types of preparations were dissolved in, and treated with varying concentrations of hydrochloric acid, and heated for different time intervals in a boiling water bath. No measurements were made of pH values after dissolving the ACTH in the acid solutions. The solutions (10 mg/ml) were then brought to pH 3 by the addition of 1 N sodium hydroxide and diluted 1:100 in isotonic saline for injection purposes. Pitressin (Parke Davis and Co.) was employed as a standard for assay purposes, the commercial preparation being diluted from the original concentration of 20 units/ml to 1, 10, or 100 milliunits/ml in isotonic saline. Tests for antidiuretic activity in these various preparations were made in the manner described by Birnie *et al.*(5), using the hydrated rat. Adult, male, Long-Evans rats, weighing 200-350 g, were fasted, but allowed access to tap water for 18 hours. They were then given 3 ml of water/100 sq. cm surface area for each of 3 successive hours. At the time of the third dose of water, the urine excretion for the previous 2 hours was measured, the test substance was injected intraperitoneally, and urine collections were begun for the 90-minute test period. The urine excreted in this 90-minute period of time was calculated as the percent of the water administered in the hydration period minus the volume excreted up to the time of administration of the test preparation. All solutions were assayed in groups of 3 or 4 animals, individually handled, accompanied by saline and/or pitressin control groups. Pressor tests were made on sodium pentobarbital anaesthetized and heparinized unfasted 400-500 g male rats by direct cannulation of the internal carotid artery using the mercury manometer. These animals exhibited no pressor reaction to the intravenous administration of physiological saline solution and showed minimal (10 mm Hg) pressor responses to 2-4 milliunits of Pitressin. The log dose-response curve was linear in the range 4 to 100 milli-

---

* Assisted by grants from the National Institutes of Health, U. S. Public Health Service, and from the Armour Laboratories and the Merck Co.

† Grateful acknowledgement is made to G. F. Hungerford, Elizabeth Swirnofsky, and Harold Papkoff for assistance rendered.

1. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, v149, 413.

2. Li, C. H., Transactions, Macy Conference on Metabolic Aspects of Convalescence, 1948, v17, 114.

3. Li, C. H. and Reinhardt, W. O. In preparation.

4. Li, C. H., *J. Am. Chem. Soc.*, in press.

5. Birnie, J. H., Eversole, W. J., Boss, W. R., Osborn, C. M., and Gaunt, R., *Endocrinol.*, 1950, v47, 1.

TABLE I. Comparison of Water Excretion by the Hydrated Rat Given Various Preparations of ACTH, Pitressin or Saline.

Preparations tested	No. of different samples tested	No. of rats	Treatment		Avg % water excreted†
			Hydrochloric acid, normality	Duration of treatment, min.	
Isotonic saline	7	21	0	0	65 (48-86)
Pitressin					
1 milliunit	3	13	0	0	52 (38-63)
10 ”	7	21	0	0	15 (6-29)
100 ”	7	19	0	0	19 (2.5-29)
ACTH protein* (A) and (C)					
1	7	31	0	0	16 (9-26)
2	1	2	.1	30	18
3	1	3	.2	30	8
4	1	3	.2	60	67
5	1	3	.2	120	60
6	2	7	.2	180	62
7	1	4	.2	360	63
8	3	15	.5	30	56 (40-76)
9	3	10	1	30	57 (51-60)
ACTH pepsin digestion mixture (B)					
10	2	12	0	0	12 (8-16)
11	1	4	.2	60	54
11	1	4	.2	120	75
11	1	4	.2	180	65
ACTH acid peptide (D)					
12	2	7	.2	60	55 (54-56)

* See text for description of preparations (A)-(D). Test injections were made intraperitoneally in 1 ml of isotonic saline, pitressin in the dose levels indicated, and ACTH preparations in 100 μg per test dose.

† Parenthetical figures indicate range of averages of 3 or 4 animals per group.

units. Test substances were injected intravenously (external jugular vein) in levels of 100 micrograms in 0.1-0.2 ml solution. Pressor responses were graded as positive or negative at the dose level employed.

Results. The results of testing 12 different ACTH preparations on 107 animals in the antidiuretic test are presented in tabular form (Table I) to compare the per cent of water excretion after various ACTH preparations as compared with saline and pitressin controls. The averages and ranges (for each group of 3 or 4 rats) are presented for the various types of preparations employed. It will be noted that the non-acid treated and unheated preparations uniformly contained at least 10 milliunits of activity per 100 micrograms as compared with the pitressin controls. No attempt was made to determine the total contamination by further titration, although it was evident that many of these preparations contained far in excess of this amount of contaminating antidiuretic activity (unreported assays). On the other hand, treatment of the ACTH protein (A) or (C), or ACTH peptides from pepsin digestion with acid and heating reduced the antidiuretic activity by a factor of at least 10. The exact figure cannot be stated because the original materials were not assayed for their total contamination. It will further be noted that pepsin digestion of protein does not reduce antidiuretic activity, whereas added treatment with acid and heat does affect this result. It will be remarked that antidiuretic activity was reduced in preparations heated either in 0.5 N HCl for 30 minutes or for at least one hour in 0.2 N HCl. This latter concentration of acid would appear to be most useful because the solutions can be neutralized to pH 3 without the formation of excess NaCl in the injection mixture.

Pressor tests were conducted on various

types of ACTH preparations. In 6 of the preparations treated with acid and heat as indicated above, pressor effects were absent in the 100 μg dose levels tested, whereas it was uniformly present in the nontreated preparations. In the instance of the pressor tests, no effort was made to assay for total original contamination. It was thought to be of importance that antidiuretic and pressor activities were found to parallel each other, both in qualitative and in quantitative tests insofar as these were conducted.

The assumption is made in these experiments that the anti-diuretic and pressor activities noted in these ACTH preparations is of posterior lobe origin. No note was made for the 90-minute intervals reported, of any distinct difference in the form of the urinary excretion curves obtained after injection of: (1) ACTH test substances; (2) pitressin; or (3) ACTH tested with added pitressin. No tests have been carried out routinely for oxytocic or chloruretic activity.

The acid and heat treatment described does not inactivate the ACTH activity of these preparations as tested by their capacity to produce lowering of the ascorbic acid level of the adrenals of the hypophysectomized rat(6). Under certain conditions, this activity may be actually enhanced by this treatment (7).

Summary. Treatment of sheep ACTH protein or peptide with 0.2 N hydrochloric acid in the boiling water bath for at least one hour, under the conditions described, produces a substantially complete reduction in antidiuretic and pressor activities originally present.

---

6. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinol.*, 1948, v42, 379.

7. Li, C. H., *J. Am. Chem. Soc.*, 1950, v72, 2815.

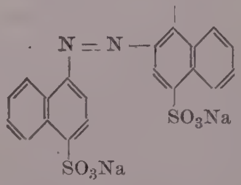
Received April 2, 1951. P.S.E.B.M., 1951, v76.

Azorubin-Binding Capacity of Normal and Pathological Sera.* (18649)

ULRICH WESTPHAL† AND PETER GEDIGK.‡ (Introduced by H. Jensen)
From the Chemical Department, Medizinische Klinik, University of Tuebingen, Germany.

The ability of serum proteins to bind certain organic dyestuffs, has been studied by means of various methods, and certain differences have been found between normal and pathological sera(1-6). A chromatographic method for the quantitative determination of the capacity of serum albumins to bind azorubin, an acidic dye, has been described by Westphal, Gedigk and Meyer(7). In the present study, this method has been used to compare the azorubin-binding capacity (ABC) of normal with that of pathological sera.

Methods. (For details see reference(7)). The azorubin (formula I) preparation used



I.

---

* The experiments described in this paper were carried out with the assistance of Mr. O. Geiss and Mr. H. Gropper.

† Present address: Army Medical Service Field Research Laboratory, Fort Knox, Ky.

‡ Present address: Medizinische Klinik der Universitaet Marburg, Marburg, Germany.

1. Bechhold, H., *Biochem. Z.*, 1907, v6, 379.

2. Friedemann, U., Oppenheimer's *Handbuch der Biochemie,* 1909, vIII, 2, 282; cf. de Haan, J. and van Creveld, S., *Biochem. Z.*, 1921, v124, 174.

3. Bennhold, H., *Z. f. d. ges. exp. Med.*, 1926, v49, 71; *Ergebn. Inn. Med. und Kinderheilk.*, 1932, v42, 273; Bennhold, H., Kylin, E., and Rusznyak, St: *Die Eiweisskoerper des Blutplasmas,* Steinkopf, Dresden, 1938, p220.

4. Ehrström, M. Ch., *Acta Med. Scand.*, 1936, v90, 427.

5. Gottlieb, H., and Ludwig, H., *Z. f. klin. Med.*, 1937, v131, 358.

6. Huggins, C., Jensen, E. V., Player, M. A., and Hospelhorn, V. D., *Cancer Research,* 1949, v9, 753.

in these studies was obtained from Hollborn und Soehne, Leipzig, Germany. Two ml of serum were mixed with azorubin, dissolved in 0.5 ml of 0.6% sodium chloride, and after standing 30 min., the mixture (pH 7.8 to 8.0) was chromatographed on aluminum oxide (preparation of E. Merck, Darmstadt, Germany, standardized according to Brockmann), which was made anionotropic by the method described previously(7). The amount of dye used was in excess of the binding capacity of the serum analysed. Three parallel runs (in some cases two), using 3 different concentrations of azorubin, were made with each serum. The azorubin concentrations in the final mixtures to be chromatographed were 100, 200, 300 or 400 mg %. During the chromatographic process, the excess azorubin, not bound to the proteins, is adsorbed in a narrow zone while that part of the dye which is combined with the proteins is carried through the column and can be estimated colorimetrically in the filtrate. All of the azorubin in the filtrate is bound to proteins, its concentration representing a relative measure of the azorubin-binding capacity of the serum tested. Using an anionotropic aluminum oxide preparation of constant activity under the same conditions, reproducible results can be obtained. In 13 triplicate and 4 duplicate experiments on human sera, for instance, using 200, 300 and 400 mg % azorubin in the mixture to be chromatographed, the observed average values ranged from 145.2 to 193.6 mg %; the maximal deviation from the average values was –3.9%, and the average maximal deviation 2.0%(8). The concentration of azorubin found in the filtrate after the chromatographic analysis will be designated "ABC observed." This value, given in mg %, refers to the serum diluted with the dye solution in the ratio of 4 : 5. As pointed out previously(7), azorubin is bound exclusively, in normal serum, to albumin as shown by electrophoretic analysis. This observation is in agreement with the finding of the ability of this serum component to combine with various acidic compounds(3,9). Electrophoretic studies demonstrated that even in sera giving extremely low ABC values (7.1 mg %, in a case of nephrotic stage of chronic glomerulonephritis) the globulin fractions did not contain any azorubin although the concentration of the azo dye in the serum mixture was 100 mg % before chromatographic adsorption(10). In order to characterize the albumins of different sera, the observed ABC values were divided by the albumin concentration. The values thus obtained will be designated "specific ABC" and will be expressed in moles of azorubin per gram albumin. It should be emphasized that these values are only relative since they are obtained by a competitive reaction between the aluminum oxide and the serum albumin, and they cannot be compared directly, therefore, with the capacity of albumin to bind acid compounds, as determined by other methods (*e.g.*, dialysis, electrophoresis)(7). Since circumstances did not permit the electrophoretic determination of the albumin concentration, the Howe procedure was employed. It is known that this latter method gives higher albumin values than the electrophoretic analysis, especially in certain pathological cases of low albumin concentration such as nephrosis(11). The pH at which the binding of azorubin to albumin is estimated, is that of the chromatographic filtrate (pH 6.2).

Experiments and results. As can be seen from Table I (Group 1), all sera of *normal persons* studied gave ABC values above 100 mg % (Class I); values under 80 mg % (Class III) were found in pathological cases only. No definite evaluation can be made for the range from 80 to 100 mg % (Class II). For the "specific ABC" values, an arbitrary value of 6.0 x 10^{-5} moles of azorubin per gram albumin was chosen as borderline between normal and pathological sera. Normal ABC values were obtained also for the sera of

---

7. Westphal, U., Gedigk, P., and Meyer, F., Hoppe-Seyler's *Z. f. physiol. Chem.*, 1950, v285, 36.

8. Geiss, O., *Inaugural-Dissertation.* Tübingen, 1948.

9. Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.*, 1946, v68, 1486, 2299.

10. Ott, H., unpublished experiments.

11. Gutman, A. B., *Advances in protein chemistry*, 1948, v4, 155, 182.

TABLE I.

Group	Diagnosis (No. of patients in parentheses)	Class and range of ABC (mg %)		ABC observed (mg %)	Specific ABC (moles × 10^{-5} per g albumin)
1. Normal	Normal persons (26)	I	>100	106 to 195	6.4 to 9.8
2. Normal as to serum proteins	Essential hypertension (16), mostly fully compensated; light cases of gastritis (5), and peptic ulcer (3); sciatica (1); colloid goitre (1)	I	>100	107.6 to 182	7 to 10.6
3. Nephrosis	Nephrotic stage of chronic glomerulonephritis (3); amyloid disease of kidney (2); lipoid nephrosis (1)	III	<80	3.2 to 78.9	1 to 8.3*
4. Liver disease	Infectious hepatitis (9); chronic infectious hepatitis (2); cirrhosis (2); enlargement of liver, splenomegaly (1). Bilirubin: 0.6 to 31.6 mg %	I	>100	101 to 163.4	7.2 to 8.3
	Infectious hepatitis, cholangitis, cholecystitis (1); chronic hepatitis (1). Bilirubin: 5.2, 2.9 mg %	II	80–100	81.8 to 88.2	5.7 to 6.2
	Homologous serum hepatitis (1); infectious hepatitis (1); coma hepaticum (1); severe hepatitis of unknown etiology (1); gastric carcinoma, liver metastases, obstructive jaundice (1). Bilirubin: 8.8 to 29.7 mg %	III	<80	16.9 to 75.7	1.3 to 4.2
5. Infectious diseases	T.b. of various locations (10); cystitis due to *E. coli* (2); pyelonephritis (1); Boeck's sarcoid (1)	I	>100	103.1 to 171.7	6 to 9.8
	T.b. of lungs (3)	II	80–100	89.4 to 95.1	6 to 6.8
	T.b. of various locations (5); broncho- and lobar pneumonia (5); brucellosis (1); chronic tetanus (1)	III	<80	22.6 to 76.7	2.5 to 6.2
6. Subacute bacterial endocarditis	Subacute bacterial endocarditis, combined aortic and mitral insufficiency (1)	II	80–100	87.8	6.5
	Subacute bacterial endocarditis, combined aortic and mitral insufficiency (3); diffuse glomerulonephritis (2); aortic insufficiency (1); mitral insufficiency (1)	III	<80	31.3 to 79.9	2.3 to 7.3
7. Neoplasm	Astrocytoma (1); cavernous hemangioma of lung (1); clear cell carcinoma of right kidney (1)	I	>100	102.2 to 132.2	6.5 to 8.5
	Chronic myelogenous leukemia (3); hypernephroma, liver metastases (1); melanosarcoma (1)	III	<80	46.1 to 73.1	3 to 6.9
8. Myeloma	Multiple myeloma (1)	I	>100	107.5	10.2
	β_2—plasmocytoma (1) γ—plasmocytoma (2)	III	<80	46.6 to 69.9	4 to 7.9

* Specific ABC value of 8.3 found during recovery; see Table II.

patients whose *serum protein patterns* are generally considered to be "*normal*" (Group 2, Table I).

In all cases of *nephrosis* (Group 3), ABC values were found to be in the lower range, and the specific ABC values were also found to be extremely low. These results confirm earlier findings of the low binding capacity of serum albumins in nephrosis(3,4). A case of chronic glomerulonephritis of primary inflammatory origin, which resulted in a nephrotic stage, was followed over a period of 33 months (Table II). Definite clinical improvement during 8 months of hospitalization was reflected by a gradual increase in the ABC. After 2 years, typical symptoms of the glomerulonephritis were found, again accompanied by a decreased specific ABC value.

TABLE II. ABC Values in a Case of Chronic Glomerulonephritis, Nephrotic Stage.

Time after admission	Clinical picture	ABC observed (mg %)	Specific ABC (moles × 10^{-5})
0*	Severe edema; ascites	15.1	3.0
6 wk		37.4	4.3
3 mo.	Retrogression of edema	56.2	6.6
8 mo.	No edema, marked improvement of clinical picture	78.9	8.3
33 mo.	Edema on both lower legs	72.0	4.6

* Admission.

In the cases of *liver disease* (Group 4, Table I), a wide range of ABC values was found. No correlation could be established between ABC and the bilirubin level, Takata-reaction or sedimentation rate. However, a definite parallelism was observed between the severity of the disease and decrease in ABC, since all 5 patients of Class III showed very heavy damage of the liver parenchyma confirmed by autopsy§ in 3 fatal cases. Among the 14 cases with normal ABC values (Group 4, Class I), on the other hand, only one patient, suffering from severe infectious hepatitis, was suspected of having major liver damage; the ABC values of this serum were found to be 103.0 mg %, 7.3 moles x 10^{-5}. No patient of Class I and II, Group 4, died.

Since the bilirubin level has been found generally to be elevated in these cases, experiments were performed in order to see whether the increased bilirubin concentration could account for the decrease in ABC values. Addition of crystalline bilirubin to normal serum resulted in a lowering of the ABC values. This effect is probably due to a combination of bilirubin with the same groups of the albumin molecules to which azorubin attaches itself(12). The amounts of bilirubin added were of the same order as observed for the sera of Class III (Group 4, Table I). The decrease in ABC, however, was found to be much smaller than the deviation of the ABC values from the borderline of 80 mg %. Only a part of the lowering effect can, therefore, be accounted for by the elevated bilirubin level.

In the group of *infectious diseases* (Group 5, Table I), parallelism was observed between lowered binding capacity and the severity of the clinical picture. The patients of Class III, 6 of whom died, again had the more unfavorable diagnosis, and the lowest ABC values were found in the sera of 4 patients tested 1 or 2 days before death. Most patients of Class I were lighter cases; clinical improvement took place in all of them, as well as in the patients of Class II. The results in this group indicate that abnormal ABC values were found whenever the disease had reached a severe "toxic" phase. In 7 out of 8 cases of *subacute bacterial endocarditis* (Group 6, Table I), low ABC values were observed. In 4 of these, liver damage, and in 3, nephrotic symptoms were found. Six patients of this group died.

In 10 cases of *neoplasms* (Group 7, Table I), ABC values were found to overlap into all 3 classes. In all patients of Class III, the illness was far advanced and severely affected their general condition. However, advanced phases of these neoplastic diseases did not coincide regularly with low ABC, since they occurred also in sera of patients of Class I and II. Death occurred in 4 patients of Class II and III. Lack of correlation between the clinical picture and ABC values was found also in 4 cases of *myeloma* (Group 8, Table I).

Examination of the sera of 21 patients with *endocrine diseases* did not reveal any abnormality in the ABC. This group included 4 cases of moderate diabetes, 5 of tetany, 2 of myxedemas, one of hyperthyroidism, one of slight adrenal insufficiency, 3 of hypophyseal metabolic disorders, and several patients suffering from obesity or emaciation of endocrine origin. In none of these patients was the course of disease alarming. No abnormality in ABC values could be observed either in 11 out of 13 cases of *heart and circulatory diseases*; the 2 patients with lowered ABC suffered from liver congestion. In other sera tested, normal ABC also was observed in 2 cases of thrombocytopenic purpura (thrombocytes 21,000 and 45,000, respectively) and in a patient with pernicious anemia treated with liver extract (campolon). Decreased ABC values (51.0 mg %; 3.7 moles x 10^{-5}) were found in the serum of a patient who died from porphyria during the second month of pregnancy. Severe damage of the liver parenchyma, as based on clinical tests, was verified by autopsy.

---

§ The autopsies were done by the Pathological Institute, University of Tuebingen, Tuebingen, Germany.

12. Westphal, U., Ott, H., and Gedigk, P., *Hoppe-Seyler's Z. f. physiol. Chem.*, 1950, v285, 200.

Discussion. The determination of the ABC is not supposed to serve as a tool of clinical diagnosis, although, in certain cases, the test may perhaps be useful for the characterization of sera as to binding function of their albumins. The estimation of the ABC is rather intended to obtain information on the quality of serum albumin, and to aid in the elucidation of the problem whether or not there are chemical differences in the serum albumin molecules of normal and pathological sera. The ABC of albumin apparently is not altered by admixture of nonbinding proteins: The observed and specific ABC values of a 1.2% solution of crystalline human albumin¶ in 0.15 m NaCl, to which 4.65% γ-Globulin was added, were the same as those of the globulin-free 1.2% albumin solution.

It was mentioned previously that the Howe procedure will overestimate the amount of albumin, particularly at low albumin concentrations as in nephrosis. The albumin values in the sera of our cases of nephrosis (Table I, Group 3) ranged from 0.9 to 4.1%, 5 out of 6 cases being 1.6% and below. The albumin concentration in the filtrate corresponding to the lowest specific ABC value observed (1.0 x 10^{-5} mol) was found to be 0.6%. Electrophoretic albumin concentrations of 5 nephrotic sera have been reported to range from 0.2 to 2.1%(11). It is possible, therefore, that the lowering of the specific ABC values found in nephrosis is partly due to the error inherent in the Howe method. Another factor which may add to the unreliability of the specific ABC values observed in nephrotic sera, is the observation that under the conditions of the competition reaction in the chromatographic adsorption, the specific ABC will decrease somewhat with decreasing albumin content (7). Lowering of the albumin concentration from 4.41 to 0.88% (before 4:5-dilution) resulted in a 23% decrease of the specific ABC (7). Experiments using crystalline human albumin¶ in NaCl solutions of various concentrations have shown likewise that a decrease of the albumin content from 4.2 to 1% is paralleled by a maximal lowering of the specific ABC of about 20%. At an albumin concentration of 0.3%, the specific ABC was decreased about 45%. In case of an extremely low albumin concentration in a nephrotic serum, this phenomenon may be an additional cause of a low specific ABC. However, in all the sera studied in the present paper, except those from nephrosis patients, albumin concentrations were higher: 4.0 to 5.6% in Groups 1 and 2, Table I; 2.0 to 4.9% in Groups 4 to 8, Table I. In the above experiments, the specific ABC of a 2% solution of crystalline human albumin was only 5% lower than that of a 4.2% solution. Decrease of specific ABC, as found in the sera of Classes II and III of Groups 4 to 8, Table I, therefore cannot be due to low albumin concentrations.

In contrast to nephrosis, in none of the other diseases studied (Table I, Groups 4 to 8) has the Howe method been found to give results similarly deviating from the electrophoretic values(11). In hepatitis serum, a moderate decline in serum albumin has been observed by the Howe method as well as in the electrophoretic analysis. In cirrhosis, excellent agreement was found between the two methods at low albumin levels. Electrophoretic values for tuberculosis sera have been described from 2.7 to 3.9%(11). The albumin values found in the sera of the tuberculosis patients of Classes II and III, Group 5, Table I, ranged from 2.3 to 4.0%.

¶ We are greatly indebted to Dr. J. T. Edsall and Dr. L. D. Wojcik, University Laboratory of Physical Chemistry, Harvard University, Boston, for kindly supplying the albumin and γ-globulin preparations.

The question whether differences in the ABC values of normal and pathological sera are caused by changes in the albumin molecules cannot be answered at the present time. The studies of Huggins and associates(6) are of interest in this respect. These authors, using equilibrium dialysis, found a decreased binding of phenolsulfonephthalein with the serum protein in 44.7% of the sera of 123 cancer patients (compare results listed in Groups 7 and 8, Table I, of the present paper). They correlated this change with a deficiency in the thermal coagulability of the albumin. Both of these abnormalities could be eliminated, however, on purification of the serum albumins by cold ethanol fractionation. The albumin fractions thus obtained from serum of cancer patients or normal serum, did not show significant differences in elementary composition, or in the content of thiol and amino groups.

In our own studies(13), the sera of 16 patients suffering from various diseases were used, showing normal as well as abnormal "observed" and "specific" ABC values (50.4 to 145.0 mg %; 3.2 to 8.2 moles x 10^{-5}). The albumin fractions were isolated, as in the studies of Huggins and co-workers, by the method of Cohn and associates(14). No proportionality was found between the specific ABC values of these albumin fractions and of the corresponding sera. No differences in the composition (arginine, histidine, tyrosine, tryptophane, and amino nitrogen) of the isolated albumin fractions could be found between sera giving normal and sera giving low ABC values. The specific ABC values for these separated albumins were found to be lower than in the original sera (2.6 to 5.6 moles x 10^{-5}). This decrease probably is due to a removal of salts by dialysis; diminishing the salt concentration has previously been shown to lead to a lowering of the ABC values in the test employed(7).

Summary. The relative capacity of normal and pathological sera to bind the acid dye, azorubin, was determined by means of a chromatographic method, using anionotropic aluminum oxide as absorbent. Subnormal azorubin-binding capacities (ABC) of serum albumins were demonstrated, in varying degrees, in nephrosis, diffuse damage of liver parenchyma, and severe toxic states of infectious diseases. In some cases of malignant neoplasms abnormally low ABC values were also observed.

---

13. Westphal, U., and Leube, I., unpublished experiments (1947-1950).

14. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

Received April 2, 1951. P.S.E.B.M., 1951, v76.

Effect of C-Reactive Protein on Normal Human Leukocytes.* (18650)

HARRISON F. WOOD. (Introduced by Maclyn McCarty)

From the Hospital of The Rockefeller Institute for Medical Research, New York City.

Tillett and Francis described the occurrence of a reaction between the sera of patients acutely ill with pneumococcal pneumonia and the somatic C polysaccharide of the pneumococcus(1). This reaction was obtainable only with acute phase serum, and was generally not demonstrable after the onset of convalescence. In later studies by Avery and Abernethy, and Avery and MacLeod, the reactive substance was shown by chemical and immunological methods to be a specific abnormal serum protein which reacted selectively to form a pre-

---

* The author wishes to thank Dr. Cynthia H. Pierce for her active interest in the work presented in this study, and takes pleasure in acknowledging the able technical assistance of Miss Helen H. Babcock.

1. Tillett, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1930, v52, 561.

cipitate with the C polysaccharide of the pneumococcus(2-4). The reaction with the polysaccharide was shown to occur only in the presence of traces of ionized calcium and to be reversed by citrate, which rendered the calcium ions unavailable to the protein-carbohydrate complex. As a result of studies carried out over the past twenty years it has become evident that the C-reactive protein is present in the serum of patients ill with a wide variety of infectious and non-infectious diseases(5,6). The concentration of this protein in the serum as determined by a precipitin reaction with specific rabbit antiserum, or alternatively by reaction with the C polysaccharide, is being used as a measure of the activity of the disease process in rheumatic fever(7). Despite the knowledge of the wide variety of illnesses which are characterized by the appearance of the C-reactive protein in the serum, there have been as yet no indications of its site or mechanism of origin or of its biological significance. The present paper describes the effect of human C-reactive protein on normal human leukocytes. Quantitative studies using the slide cell technic of Martin, Pierce, Middlebrook and Dubos are described(8).

Materials and methods. 1. Acute phase sera from rheumatic fever patients were used as specimens containing high titers of C-reactive protein. Convalescent serum from one of these patients was used as a control. 2. Two preparations of human C-reactive protein, each isolated and purified in a different way, were used. In Preparation A, the isolation and purification of the protein from ascitic fluid were carried out with the use of pneumococcal C carbohydrate by a method essentially the same as that described by McCarty(9), except that crystallization was not carried out. The final solution of purified Preparation A had a concentration of C-protein of approximately 4%. In Preparation B, use was made of the property of calcium precipitability of C-protein as it occurs in blood serum. The procedure used in isolating and purifying this preparation is described in detail by MacLeod and Avery(3). This second preparation which had a concentration of approximately 0.2% C-protein was used in order to eliminate the possible effect on normal human leukocytes of the C carbohydrate and the citrate needed to keep the C-protein-C carbohydrate complex in solution. The concentration of C-protein contained in this and in the various other preparations used was determined by a quantitative spectrophotometric method(10).

3. The blood used for the determinations described in this study was that of one donor. The reproducibility of the observations reported was verified on blood from 4 additional donors. Immediately after being drawn, 6 cc of blood was put in a chilled tube containing 0.04 cc of a 200 mg % solution of heparin in physiological saline. The concentration of heparin used was at least 5 times less than the concentration toxic for human leukocytes. All work with the blood up to the time of incubation was done in a cold room at 4°C.

4. Slide cells, as described by Martin, Pierce, Middlebrook and Dubos, were used in all determinations. These cells consist of a cover slip sealed to a microscopic slide by a U-shaped piece of filter paper impregnated with paraffin. This structure produces a small, wide, flat chamber, which is filled with blood by capillary action and which after centrifugation permits clear visualization of the buffy coat between a red cell and a plasma layer. Accurate microscopic observations can be made of the migration of leukocytes from the buffy coat into the plasma layer after incubation. All determinations described in this paper were carried out on a mixture of 10

---

2. Abernethy, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, v73, 173.

3. MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, v73, 183.

4. MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, v73, 191.

5. Ash, R., *J. Infect. Dis.*, 1933, v53, 89.

6. Hedlund, P., *Acta Med. Scandinav. Supplement*, 1947, v196, 579.

7. Anderson, H. C., and McCarty, M., *Am. J. Med.*, 1950, v8, 445.

8. Martin, S. P., Pierce, C. H., Middlebrook, G., and Dubos, R., *J. Exp. Med.*, 1950, v91, 381.

9. McCarty, M., *J. Exp. Med.*, 1947, 85, 491.

10. Wood, H. F., and McCarty, M., In press.

TABLE I. Effect of C-protein in Acute Phase Human Serum on Migration of Normal Human Leukocytes.

Rheumatic fever patient	Greatest distance of leukocytes from buffy coat	
	1:2 dilution of untreated serum, mm	Serum treated with C carbohydrate, mm
B	.50	.25
S	.65	.25

TABLE II. Effect of 3 Concentrations of Preparation A on Normal Human Leukocytes.

Final conc. of Preparation A, mg/cc	Leukocyte reaction
.04	Destruction of leukocytes
.015	Early migration (<1 hr) followed by destruction of cells
.004	Leukocytes migrated .50 mm
Citrate-saline control	Leukocytes migrated .25 mm

parts of blood to one part of test solution prepared immediately before centrifugation and incubation. The greatest distance of migration of leukocytes from the buffy coat was determined by both macroscopic and microscopic examination of each slide cell after incubation at 37°C for one hour.

Results. 1. *Effect of acute phase sera containing C-protein.* The effect of acute phase sera containing C-protein on the activity of normal human leukocytes was tested in the following way. To 0.4 cc of serum containing approximately 0.23 mg/cc of C-protein from 2 patients with acute rheumatic fever, S.B. and L.S., was added 0.4 cc of 0.1 mg/cc C carbohydrate. The C carbohydrate removed all but a trace of the C-reactive protein. The effect of these treated sera was compared in slide cells with that of the 1:2 dilutions of their corresponding untreated sera as controls. As will be seen in Table I, the sera containing C-protein stimulated the leukocytes to migrate twice as far as did the same sera after the C-protein had been removed. A comparable difference in degree of migration was observed when an acute phase serum containing 0.35 mg/cc of C-protein was compared with a convalescent serum containing no C-protein from the same patient. These 2 sera were tested in slide cells at the same time. There was twice as much leukocyte migration in the preparations containing acute phase serum as in the control preparations in which convalescent serum was used.

2. *Effect of C-protein isolated by use of the C carbohydrate.* Preparation A was set up in slide cells in final concentrations ranging from 0.04 to 0.004 mg/cc. The microscopic results obtained with this protein preparation are shown in Table II, where it is seen that the preparation containing approximately 0.004 mg/cc stimulated the leukocytes to 2 times the migration observed in the control cells. The higher concentrations of C-protein tested destroyed the leukocytes. To determine whether or not the C carbohydrate present in the protein preparation contributed to the increased leukocyte migration, slide cells were set up with 0.1 and 0.2 mg/cc C carbohydrate. Slide cells containing normal blood alone served as controls. No evidence of increased leukocyte migration was seen in the preparations containing carbohydrate alone.

3. *Effect of C-protein isolated and purified by calcium precipitation.* To demonstrate further that C carbohydrate, citrate, or calcium present in the first preparation tested were not causing the increased migration of the leukocytes, a serologically active solution containing approximately 0.2 mg/cc of purified C-protein in saline (Preparation B), with a trace of normal human serum protein as the only known contaminating substance or substances, was used. Solutions of Preparation B ranging from 0.2 to 0.04 mg/cc C-protein were set up in slide cells concurrently with cells filled with untreated blood. After incubation for one hour, the results were as seen in Table III. Again the higher concentrations caused destruction of cells, while at lower concentrations an increase in migration was observed. In order to determine whether the trace of normal human serum protein present in the preparation caused the increase in leukocyte migration, the C-reactive protein was removed by the addition of 3 parts of 0.2 mg/cc of C carbohydrate with a trace of calcium to one part of the undiluted pro-

TABLE III. Effect of C-protein Preparation B on Normal Human Leukocytes.

Final conc. of Preparation B, mg/cc	Greatest distance of leukocytes from buffy coat, mm
.02	.6 (with destruction of cells)
.009	.7
.004	.9
Normal blood control	.4

tein preparation. In comparison with control slides filled with normal blood, no difference in degree of migration was caused by the addition of this preparation which still contained the trace of normal human serum protein originally present.

4. *Protective action of serum.* Although both acute phase serum containing C-protein and the purified isolated protein caused increased migration of the leukocytes in the test system, it was apparent that a far lower concentration of the purified preparation than of serum C-protein was required to obtain the effect, and that the purified protein was more toxic to normal leukocytes than was acute phase serum. Whereas as much as 0.1 cc of an 0.3 mg/cc solution of C-protein in serum stimulated the leukocytes in the test system without destroying them, 0.1 cc of an 0.054 mg/cc solution of C-protein isolated from serum destroyed them. In order to clarify this apparent contradiction, the following experiments were done. A sample of serum containing approximately 0.3 mg/cc of C-protein was employed. The C-protein was removed from an 0.5 cc portion by the addition of 0.5 cc of 0.1 mg/cc C carbohydrate. The protein-carbohydrate precipitate was washed in 0.01% calcium chloride. The untreated serum, the isolated C-reactive protein, and the supernatant from the carbohydrate-treated serum were tested for their effect on leukocytes. It was found that the C-protein isolated from the serum caused destruction of leukocytes, while the same amount of C-protein contained in serum enhanced migration. The supernate from the carbohydrate-treated serum had no visible effect on the leukocytes. The apparent protective action of serum against the effect of C-protein on leukocytes seen in this experiment was further tested by incubating normal human serum with Preparation B to give a C-protein concentration of approximately 0.23 mg/cc of serum. The serum C-protein mixture was then tested in comparison with an equivalent amount of C-protein in saline. The protective effect of serum was also seen in the results of this experiment, where the C-protein in serum enhanced leukocyte migration, while the same concentration of the protein in citrate and saline destroyed them.

5. *Effect of differences in the rates of migration of leukocytes from different normal donors.* In the course of the studies described in this paper, confirmation was obtained that the rate of leukocyte migration of the blood varies with each normal donor(11). In the one hour period of observation employed, the greatest distance of migration of normal unstimulated leukocytes varied between 0.1 mm for the cells of one donor to 1.0 mm for those of another. Moreover, the rate of migration exhibited by normal leukocytes has been shown by other workers to be both characteristic and constant for any individual over a period of years. The work described in this paper was done on cells which consistently showed good motility in the lower range of normal leukocyte migration. Determinations, not described in detail here, were done also on blood from donors whose leukocytes normally showed maximal migration. It was found that the cells from such individuals were stimulated to increased migratory activity by a lower final concentration (0.002 mg/cc) of purified C-reactive protein than were those of the standard blood described above. It was found also that a final concentration of purified C-protein which was stimulatory to the standard test system blood (approximately 0.004 mg/cc) destroyed the leukocytes with a normally high rate of migration. In view of these facts, it is evident that the toxic and the stimulating concentrations of purified C-protein must be determined for the leukocytes of each different normal blood to be used in the test system.

Conclusions and summary. On the basis of

---

11. Draper, G., Ramsey, H. J., and Dupertuis, C. W., *J. Clin. Inv.*, 1944, v23, 864.

studies using the slide cell technic to determine the effect of human C-reactive protein on normal human leukocytes, the following conclusions are indicated. 1. Purified human C-reactive protein in final concentrations higher than 0.005 mg/cc destroys normal human leukocytes. 2. Purified human C-reactive protein in lower concentrations stimulates the migration of normal human leukocytes. 3. C carbohydrate, sodium citrate, and normal human serum protein do not stimulate normal human leukocytes in the concentrations employed in this study. 4. The acute phase human sera tested caused increased migration of normal human leukocytes by virtue of their content of C-reactive protein. 5. Human serum diminishes at least in part the toxic effect of the C-reactive protein on normal human leukocytes.

Received April 5, 1951. P.S.E.B.M., 1951, v76.

Acute Toxicity of Veratrum Derivatives.* (18651)

EDWARD D. SWISS AND R. O. BAUER. (Introduced by G. L. Maison)
From the Department of Pharmacology, Boston University School of Medicine.

Interest in veratrum preparations has varied considerably over a period of years. Fruitful clinical application of the blood pressure lowering powers of veratrum derivatives depends on the use of materials of constant hypotensive power in the form of pure alkaloids or of mixtures standardized by their ability to lower mammalian blood pressure. The interrelationship between lethality of these substances and their hypotensive property has attracted interest as a possible method of assay(1). The results detailed below should do much to eliminate from further consideration lethality as a method of assay. While some data of the acute toxicity of veratrum derivatives have been published (1-3), strictly comparable toxicity data for the various substances have not been found. This study of the acute toxicity of veratrum preparations has been divided into (a) comparison of the acute toxicity of pure ester alkaloids or of alkaloidal mixtures from veratrum with their hypotensive potency(4). (b) Elucidation of influence of species of animal and of route of administration on the acute toxicity of a clinical veratrum preparation (Veriloid†).

Method. Albino Swiss mice (15-25 g) and albino Wistar rats (175-245 g) were weighed to the nearest 0.1 g. The solutions to be injected were prepared so as to hydrate the mouse at a rate of 0.01 ml and the rat at 0.005 ml per g of body weight. The injected animals were observed for 24 hours (actually all deaths occurred within the first hour). The New Zealand albino rabbits (2-3 kg), and the adult mongrel dogs (3-12 kg) were hydrated at the rate of 0.5 ml per kg and observed for 24 hours. The solutions of the pure alkaloids‡ were prepared by dissolving the alkaloids in a minimum amount of 5% acetic acid and diluting to the desired concen-

---

* Grateful acknowledgement is made for financial support of this work received from Riker Laboratories, Los Angeles, Calif.

1. Rowe, L. W., *J. Am. Pharm. A.*, 1925, v14, 24.

2. Christensen, B. V. and McLean, A. P., *J. Am. Pharm.*, 1936, v24, 414.

3. Krayer, O., Acheson, G. F., *Physiol. Rev.*, 1946, v26, 283.

4. Maison, G. L., Gotz, E., and Stutzman, J. W., *J. Pharm. and Exp. Therap. Proc.*, 1951, v101, 24.

† *Veriloid* (Trademark of Riker Laboratories) is a biologically standardized alkaloidal extract of *Veratrum viride* of uniform hypotensive potency.

‡ The substances utilized were kindly provided by the following sources: Eli Lilly and Company, Ind., Protoveratrine; Merck and Company, Rahway, N. J., Veratrine; S. B. Penick and Company, Detroit, Mich., Veratrine; Dr. S. J. Ringel, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, Beltsville, Md., Cevadine; Riker Laboratories, Los Angeles, Calif., Protoveratrine, Veriloid and Veratridine; Squibb Institute for Medical Research, New Brunswick, N. J., Germitrine, Neogermitrine, and Germidine.

TABLE I. Intraperitoneal Toxicity of Veratrum Alkaloids and Extracts.

Drug Lot # (MP °C)	LD_{50} C.L. 19/20 mg/kg	No. of animals tested	Standard hypotensive dose, µg/kg/min.*	LD_{50} in terms of standard reference potency†
Neogermitrine JF 115—C_3 (231-232°)	.51 (.41-.63)	40	.115	4.43
Protoveratrine (Riker) 7/18/50 (265°)	.44 (.30-.64)	40	.212	2.07
Protoveratrine (Lilly) P—49366 (246°)	.37 (.29-.48)	110	.218	1.72
Germidine JF 68—C 8 (230-232°)	10.0 (9.1-11.0)	29	.416	24.0
Veratridine 5/19/50 (171°)	1.35 (1.12-1.62)	70	2.13	0.63
Cevadine‡ (209°)	3.5 (2.7-4.4)	50	5.55	0.63
Standard reference powder (Veriloid)	3.75 (3.4-4.1)	390	1.00	3.75
Veratrine§ (Penick) (NF IV)	8.5 (7.5-9.6)	50	3.7	2.3
Veratrine§ (Merck) (145-155°)	7.5 (6.1-9.2)	30	3.7	2.5
Veratrone	2.45 (1.8-3.2)‖	48	0.85	2.9
Fluid extr. USP IX	182 (151.6-218.4)¶	30	42.3	4.3

* These data are from Maison, Gotz and Stutzman(4). Standard dose is an estimate of µg/kg/min. over 10-minute period required to produce average fall of mean arterial pressure of 30% in a large number of anesthetized dogs.

‖ Calculated on basis that 100 ml of Veratrone equals ¼ g.

† Calculated from: LD_{50} value divided by the hypotensive potency in standard dose.

§ Veratrine is a mixture of alkaloids (predominantly cevadine and veratridine) obtained from the seeds of sabadilla.

¶ Calculated on basis that 1 ml of extract equals 1 g crude root.

‡ Tiglic acid ester of cevine.

tration with physiologic saline. The various purified extracts of veratrum were dissolved in a minimum of a solution of equal parts of propylene glycol and alcohol§ and diluted to the desired concentrations with acidified physiologic saline. All determinations were calculated by the method of Litchfield and Wilcoxon(5). Care was taken to utilize enough animals in each determination to produce statistical validity as reflected in the Chi² value.

Results. The acute toxicities of several pure veratrum alkaloids and their hypotensive potencies are listed in Table I. Fig. 1 depicts toxicity versus depressor power for a series of veratrum extracts of the same general nature at various stages of purification. Each point represents the lethal dose 50 (LD_{50}) determination for a different extract. Table II presents the acute toxicity values for *Veriloid*† standard reference powder in various species as well as by different routes of administration. Also included in this table is a demonstration of the validity of the method shown by repeated determinations of the toxicity of a particular powdered extract.

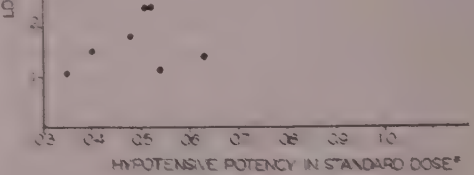


FIG. 1.
Various purified extracts of *Veratrum viride*.

---

§ Final concentration of alcohol in injected solution never exceeded 2%.

5. Litchfield, J. T. and Wilcoxon, F., *J. Pharm. and Exp. Therap.*, 1949, v96, 99.

* Standard dose is an estimate of µg/kg/min. over a 10-minute period required to produce an average fall of mean arterial pressure of 30% in a large number of anesthetized dogs.

TABLE II. Acute Toxicity of Veriloid Standard Reference Powder.

Animal	Route	LD_{50} 19/20 C.L., mg/kg	Date	No. of animals tested
Mice	Oral	4.5 (3.0-6.84)		40
”	I.P.	3.4 (2.6-4.6)	4/3/50	60
”	”	3.45 (2.83-4.21)	8/18/50	40
”	”	2.95 (2.54-3.42)	8/17/50	30
”	”	3.3 (2.8-3.83)	8/23/50	40
”	”	3.0 (2.5-3.57)	8/30/50	40
”	”	4.4 (3.7-5.2)	1/8/51	60
”	”	4.3 (3.5-5.2)	1/8/51	60
”	”	4.25 (3.6-4.9)	1/9/51	30
”	”	4.1 (3.2-5.2)	1/10/51	60
”	I.V.	0.95 (0.81-1.12)	—	40
Rat	Oral	12.2 (10.9-13.8)	—	291
”	I.P.	1.69 (1.26-2.26)	—	25
”	I.V.	0.44 (0.39-0.48)	—	60
Rabbit	Oral	18.7 (15.4-22.6)	—	63
Rabbit	I.V.	0.27 (0.24-0.31)	—	53
Dog	”	Emesis occurred—no death		

Discussion. Mean lethal dose (M.L.D.) values (intraperitoneal mice) were determined for *Veratrone*[¶] in mice by Rowe(1) and by Christensen and McLean(2). Their results fall within the confidence limits obtained in this investigation. The nine determinations of the acute toxicity of *Veriloid*[†] standard reference powder (Table II) show a greater consistency of results than was to be expected from the data of previous workers. A seasonal variation was observed, with the less toxic results being obtained in the winter months. The oral lethal dose for the dog was not obtained because emesis occurred.

The hypotensive potencies of the various substances as revealed by the dog assay method have been utilized for comparative purposes in this study. While there may be argument as to the limit of accuracy of these data they do offer the best estimate available for the purpose.

The results presented in Table I suggest that no correlation existed between the acute toxicity and the hypotensive potencies of the pure veratrum alkaloids. Although a rough correspondence between hypotensive potency and acute toxicity existed for the extracts reported in Fig. 1, predictability of hypotensive power was not indicated for the acute toxicity method.

Summary. (1) The acute toxicity in mice by the intraperitoneal route for several pure veratrum alkaloids and several alkaloidal mixtures from veratrum are presented. (2) The results suggested that predictive correlation did not occur between acute toxicity and hypotensive potency. (3) The influence of route of administration and species of animal on lethal dosage was demonstrated. Oral administration did not produce lethality in species capable of emesis.

Received April 10, 1951. P.S.E.B.M., 1951, v76

[¶] Veratrone (Trademark Parke Davis and Co.) is a nonalcoholic solution equal to ¼ the strength of fluid extract Veratrum viride by alkaloidal assay (chemical).

Demonstration of Glycogen in the Human Liver by the Electron Microscope. (18652)

Councilman Morgan and Robert W. Mowry. (Introduced by R. W. G. Wyckoff)
From the Laboratory of Physical Biology and Laboratory of Pathology and Pharmacology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

The purposes of this paper are to show that glycogen is readily visible in thin tissue sections examined by the electron microscope and to suggest that its removal by diastase offers a convenient way of differentiating it from other cellular constituents since differential staining of electron microscopic preparations has so far been unsuccessful.

Procedure. Thin blocks of human liver were fixed in alcohol-formalin (10% formalin in 95% alcohol), known to be an excellent fixing agent for glycogen(1). After fixation the blocks were dehydrated in absolute alcohol, cleared in xylol and embedded in paraffin. Sections were stained for glycogen by Best's carmine stain, the Bauer reaction and the periodic acid-leucofuchsin method and examined under the light microscope(2). From livers showing abundant glycogen small blocks were excised from the paraffin embedded tissue and the paraffin removed by xylol followed by acetone. The tissue was then embedded in methacrylate and sectioned by the methods outlined by Newman, Borysko and Swerdlow(3) except that a glass edge(4) was used in place of a steel knife. Thin sections were floated onto formvar coated screens and the methacrylate removed by amyl acetate. The sections were shadowed with palladium. All observations were made with an RCA type EMU electron microscope.

To determine that glycogen was not lost during methacrylate embedding sections one and five μ thick were cut from each methacrylate block mounted on glass slides and stained for glycogen. Examination in the optical microscope showed that the reembedding had not appreciably altered the tissue and that as much glycogen was visible within the nuclei and cytoplasm as in sections taken from the original paraffin block. It should be emphasized at this point that poor preservation of cellular structure, largely resulting from post-mortem autolysis, is more evident in thin sections. In the light microscope 5 μ thick sections show what one is accustomed to consider as well preserved liver. In progressively thinner sections superimposition of structures diminishes and clumping and distortion of the cytoplasm become more and more striking.

In the electron microscope many nuclei are shown to contain varying amounts of a moderately dense substance having the form and distribution of glycogen as previously described(5) and especially as seen in stained, one μ thick sections in the optical microscope. The nuclei are generally enlarged and the chromatin is concentrated at the periphery. In the majority of nuclei the material presumed to be glycogen aggregates as small globules at the periphery (Fig. 1). Less commonly it forms an incomplete coating on the nuclear membrane and encloses irregular particles of similar density (Fig. 2). Rarely is the nucleus largely filled (Fig. 3).

In occasional nuclei small globules of apparent glycogen are enveloped by very dense, irregular material resembling chromatin (Fig. 4). Since in such nuclei no other structures can be identified as nucleoli and since they are of similar size it is possible that these may be glycogen filled nucleoli. In the cytoplasm of cells from blocks of tissue shown to contain granules of cytoplasmic glycogen in the optical microscope irregular, sharply demarcated particles of amorphous material similar in density to that seen within the nuclei are

---

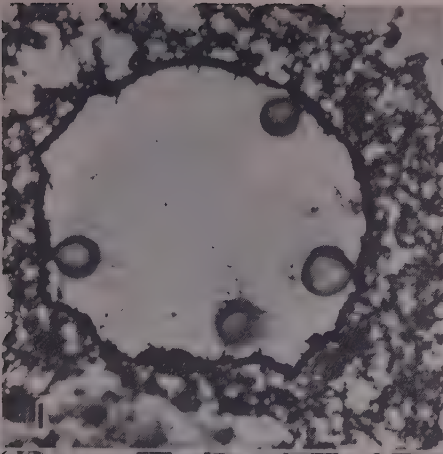
1. Lillie, R. D., *Bull. Internat. Med. Museums,* 1947, v27, 23.

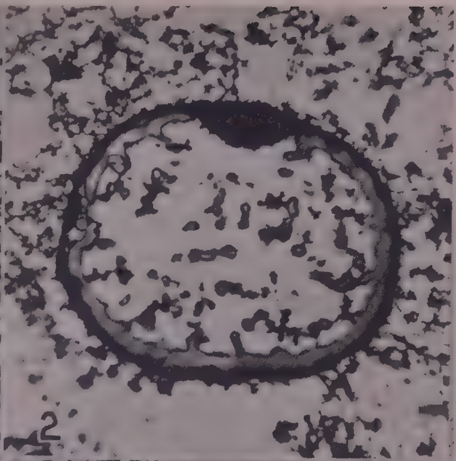
2. Lillie, R. D., *Histopathologic Technic,* The Blakiston Co., Phila., 1948.

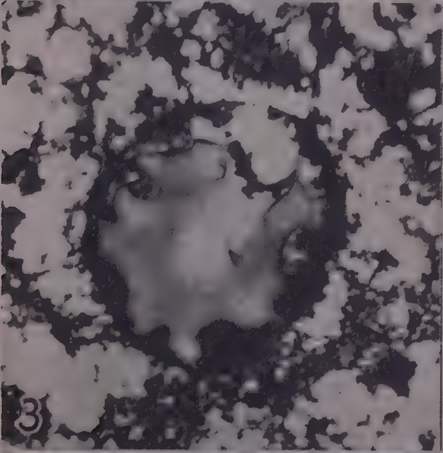
3. Newman, S. B., Borysko, E. and Swerdlow, M., *Science,* 1949, v110, 66.

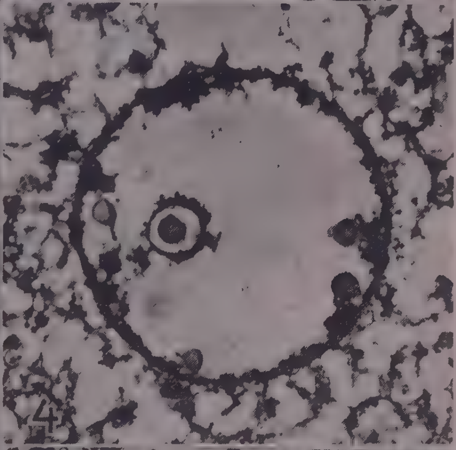
4. Latta, Harrison and Hartman, J. Francis, Proc. Soc. Exp. Biol. and Med., 1950, v74, 436.

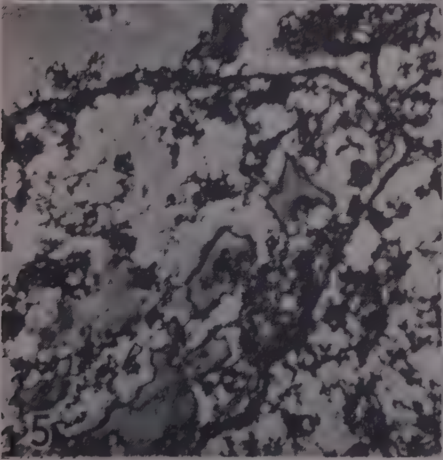
5. Chipps, H. D. and Duff, G. C., *Am. J. Path.,* 1942, v18, 645.











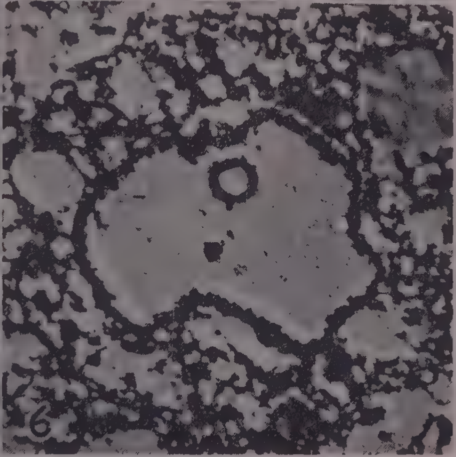


FIG. 1. Liver cell nucleus containing 4 glycogen droplets. Magnification = 3,466×.

FIG. 2. Intranuclear glycogen, much of which adheres to the nuclear membrane. Magnificaiton = 3,866×.

FIG. 3. Nucleus largely filled by glycogen. Magnification = 6,000×.

FIG. 4. Nucleus containing 5 droplets of glycogen, one of which lies within a possible nucleolus. Magnification = 5,200×.

FIG. 5. Cytoplasm containing irregular, sharply circumscribed, moderately dense masses resembling glycogen in nuclei. Magnification = 2,933×.

FIG. 6. Typical nucleus in duplicate section after diastase digestion. Magnification = 4,800×.

observed (Fig. 5).

To determine whether diastase affects this material 5 μ, 1 μ and ultrathin sections were incubated at 37°C in 0.1% USP IX malt diastase in Lillie's pH 6.0 saline phosphate buffer for 1 hour(6,7). Examination of stained sections in the light microscope as well as ultrathin sections in the electron microscope reveal complete removal of the presumed nuclear and cytoplasmic glycogen. Often in nuclei a faint ring is left on the formvar film, suggesting the outline of a pre-existing deposit of glycogen (Fig. 6). Occurring in the cytoplasm are masses of material which are poorly demarcated, larger and uniformly less dense than the presumed glycogen particles (best seen in Fig. 5 and 6). These are not removed by diastase. Other cytologic features appear unaltered. Sections incubated in the buffer solvent without diastase show no effect upon the glycogen. Because of the ribonuclease-like activity demonstrated after prolonged incubation of sections in malt diastase, and apparently due to contaminating traces of ribonuclease, duplicate sections were incubated in a solution of crystalline ribonuclease (Armour's, bovine pancreas) with control sections of pancreas (8). No removal of material regarded as nuclear or cytoplasmic glycogen is observed in the electron microscope.

Summary. In sections of human liver examined by the electron microscope, material is visible in the nuclei and cytoplasm, which, because of its resemblance in morphology and location to glycogen seen in stained sections examined in the light microscope and because of its removal by diastase, is presumed to be glycogen.

---

6. Lillie, R. D. and Greco, J., *Stain Technol.*, 1947, v22, 67.

7. Lillie, R. D., Greco, J. and Laskey, A., *Anat. Rec.*, 1949, v103, 635.

8. Lillie, R. D., *Anat. Rec.*, 1949, v103, 611.

Received March 9, 1951. P.S.E.B.M., 1951, v76.

Hemolysin from the Yeastlike Phases of Some Pathogenic Fungi. (18653)

S. B. SALVIN. (Introduced by C. L. Larson)

From the Rocky Mountain Laboratory (Hamilton, Mont.), National Microbiological Institute, National Institutes of Health, Public Health Service.

The only hemolysin previously described in a pathogenic fungus was isolated from the ground mycelium of *Aspergillus fumigatus*(1). The present investigation is concerned with an hemolysin or hemolysins from the yeastlike phases of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Candida albicans*, and *Cryptococcus neoformans*.

The fungi used were from the collection maintained in this laboratory. *H. capsulatum* (strain 6515) and *B. dermatitidis* (strain 6046) were grown for 5 days in flasks containing an amino-acid broth at 37°C, and kept in constant rotation during this period of growth (2). *C. albicans* (strain 3148) and *C. neoformans* (strain 3715) were grown for 7-10 days at 25°C in a similar amino-acid broth in 1-liter Blake bottles(3). The cells, after exposure to 0.5% formalin for 72 hours, were thoroughly washed in saline at 3°-5°C, and stored in 1:10,000 merthiolate until ready to

---

1. Henrici, A. T., *J. Immunol.*, 1939, v36, 319.

2. Salvin, S. B., *J. Bact.*, 1950, v59, 312.

3. Salvin, S. B., *J. Immunol.*, 1950, v65, 617.

TABLE I. Properties of Soluble Supernates from Four Pathogenic Fungi.

	Dry wt (after dialysis), mg/ml	Kjeldahl N, mg/ml	Total carbohydrate, mg/ml (Molisch)
(6046) *B. dermatitidis*	3	.22	.32
(6515) *H. capsulatum*	10	.46	.30
(3148) *C. albicans*	1.8	0	.66
(3715) *C. neoformans*	1.8	0	1.02

be dissociated. Eight ml aliquots of concentrated cell suspensions were vibrated with 10 g of glass beads* in a container of a Mickle tissue disintegrator† for 5-6 minutes at 4°C, and the resulting suspension centrifuged at 4°C for one-half hour at 40,000 r.p.m. The resulting supernates from typical lots of the four fungi studied had the characteristics shown in Table I after prolonged dialysis in redistilled water at 4°C. In attempts at hemagglutination, 0.5 ml of each supernate was added to an equal amount of a 0.25% suspension of erythrocytes of hen, guinea pig, rabbit, human (types O and AB), goat, mouse, rat, sheep, and horse. No distinct hemagglutination was observed; subsequently the concentration of erythrocytes was varied from 0.1% to 1%, and the time of incubation from 0.5 to 3 hours, both at room temperature and at 37°C. No marked hemagglutination was obtained.

In the hemolysin tests, 0.5 ml of a 1% cell suspension was exposed to the supernate for 3 hours at 37°C, then allowed to remain overnight at 5°C before readings were made. Experiments on several types of erythrocytes showed the best hemolysis characteristically on cells from hens and guinea pigs (Table II). Accordingly, in the studies on the effects of environmental factors on the action of the fungal hemolysin, guinea pig or hen cells were employed.

To determine the influence of pH on hemolysis, the supernates from *B. dermatitidis* and *H. capsulatum* were dialyzed for 48 hours at 5°C against a Na_2HPO_4-NaH_2PO_4 buffer of 0.1 ionic strength and the action of the resulting solutions studied on the hemolysis of guinea pig erythrocytes (Table III). At pH's of 6.0 and below, the controls in buffer produced hemolysis. The highest significant hemolytic activity occurred at pH 6.5 and the optimum point for differentiating *B. dermatitidis* and *H. capsulatum* at pH 7.0.

The ionic strength of this Na_2HPO_4-

TABLE II. Species Differences in Erythrocytes on Hemolysis by Supernates from the Yeast-like Phases of *B. dermatitidis* and *H. capsulatum*.§

		Dilutions of antigens					
		2	4	8	16	32	64
Guinea pig	Bd	*	*	†	‡		
	He	*	*	*	†	†	‡
Hen	Bd	‡					
	He	*	*	*	†	‡	
Horse	Bd	‡					
	He	*	*	†	‡		
Mouse	Bd	†	†	‡			
	He	†	†	†	‡		
Rat	Bd	‡					
	He	*	†	‡			
Sheep	Bd	‡					
	He	‡					
Human "O"	Bd	‡					
and "AB"	He	‡					

Bd = Supernate from *B. dermatitidis*.
He = Supernate from *H. capsulatum*.
* Complete hemolysis.
† Partial hemolysis.
‡ No hemolysis.
§ Goat not included because cells in diluent (minus supernate) hemolyzed.

Note: Controls with broth, broth treated with formalin (as in preparation of antigen), saline, and PO_4 buffer (of 0.1 ionic strength and at pH 7) produced no hemolysis with any of the erythrocytes.

TABLE III. Effect of pH on Hemolysis of Guinea Pig Erythrocytes.

	pH*				
	6.0	6.5	7.0	8.3	Saline
B. dermatitidis supernate	>128†	64	8	4	4
H. capsulatum supernate	>128	64	32	4	32

* All buffers were at 0.1 ionic strength.
† Titer denotes dilution of antigen producing partial or complete hemolysis.

---

* Type 511—Pavement Marking Beads, Minn. Mining and Mfg. Co., St. Paul, Minn.

† Mickle tissue disintegrator. H. Mickle Co., Hampton, England.

TABLE IV. Influence of Ionic Strength (μ) on Hemolysis of Guinea Pig Erythrocytes.

Supernates from	.05	.1	.15	.2	.4
B. dermatitidis	32	32	16	8	4
H. capsulatum	64	64	64	32	16

NaH_2PO_4 buffer was varied at pH 6.7 from 0.05 to 0.4, and the effect on hemolysis of guinea pig cells examined (Table IV).

An ionic strength of 0.1 was therefore used for future experiments.

The hemolysins of *B. dermatitidis* and *H. capsulatum* lost no activity after exposure to 60°C for 30 minutes, but were destroyed after autoclaving for 15 minutes at 15 lb. pressure.

C. albicans and *C. neoformans* hemolyzed guinea pig erythrocytes in supernate dilutions of 1:4 and 1:2 respectively, when buffered at pH 6.7. This action seemed especially interesting since these two fungus supernates had no detectable nitrogen (Table I), but had the highest carbohydrate concentration. In addition, since *H. capsulatum* supernate had the highest nitrogen content and the highest hemolysin activity, the probability presents itself that the hemolysin is a protein or protein-like substance.

Attempts were made to detect specific antibodies (both human and rabbit) by absorption of the hemolysin with specific immune serum. Thus far, however, the test has not proved of value because of the strong hemolysin-absorption properties of "normal" rabbit and human sera.

Summary. One or more hemolysins were demonstrated in soluble extracts prepared from the yeastlike phases of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Candida albicans*, and *Cryptococcus neoformans*. The hemolysin was most active against hen and guinea pig erythrocytes. Some properties of the hemolysin were investigated.

Received April 10, 1951. P.S.E.B.M., 1951, v76.

Local Effects of Cortisone on Granulation Tissue and the Role of Denervation and Ischemia.* (18654)

ROGER SHAPIRO, BERNARD TAYLOR, AND MATTHEW TAUBENHAUS.

From the Departments of Metabolic and Endocrine Research, and of Pathology, Medical Research Institute, Michael Reese Hospital, Chicago.

The effect of cortisone upon connective tissue structures has been well established. All the elements of such tissues seem to be influenced by this steroid(1-10). The mechanisms, by which cortisone acts upon connective tissue structures, are unknown. In the light of our present knowledge, a number of possible mechanisms warranted investigation. It has been well established that C_{11} oxysteroids have a catabolic effect on tissue proteins(11). It therefore seemed possible that the inhibition of granulation

---

* Aided by a grant from the National Institutes of Health, U. S. Public Health Service (R.G. 2522) to the Rheumatoid Arthritis Research Group of this Hospital. (S. Soskin, Chairman)

1. Taubenhaus, M., and Amromin, G. D., *J. Lab. and Clin. Med.*, 1950, v36, 7.

2. Ragan, C., Howes, E. L., Plotz, C. M., Meyer, K., Blunt, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 718.

3. Spain, D. M., Molomut, M., and Haber, A., *Science*, 1950, v112, 335.

4. Opsahl, J. C., White, A., Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1061.

5. Opsahl, J. C., *Yale J. Biol. and Med.*, 1949, v21, 255.

6. Seifter, J., Baeder, D. H., Dervinis, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 136.

7. Schuman, C. R., and Finestone, A. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v156, 429.

8. Benditt, E. P., Schiller, S., Wong, H., Dorfman, A., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 782.

9. Castor, C. W., and Baker, B. L., *Endocrinol.*, 1950, v47, 234.

10. Taubenhaus, M., and Lev, M., *A.M.A. Arch. Int. Med.*, in press.

tissue formation might be simply the result of lack of available protein material. Another possible mechanism was suggested when it became known that C_{11} steroids exert an effect upon vasomotor mechanisms(12). This effect may underlie the changes in granulation tissue observed. A third possible mechanism of action had to be considered, namely, the effect of altered transmission of nervous impulses, as it has been shown that cortisone profoundly influences the metabolism and function of the central nervous system(13) and the acetyl choline metabolism of peripheral nerves(14). Here again there may be some relationship between altered nervous transmission and the morphologic changes in connective tissues. The purpose of the work to be presented was to investigate the three possible inter-relationships.

Methods and results. Granulation tissue forming around turpentine abscesses in white male rats of the Sprague Dawley strain was examined histologically in the same manner as previously described(15). The first series of experiments was designed to determine whether cortisone exerts its action locally. One-half cubic centimeter of turpentine mixed with 1 mg of cortisone was injected subcutaneously into the right suprascapular region of 5 animals as a control procedure, the same amount of turpentine mixed with 1 mg of cholesterol was injected into the left suprascapular region of the same animals. After 4 days the animals were sacrificed, the abscesses removed and fixed in 10% formalin, and hematoxylin-eosin and Van Gieson sections were studied.

The leukocytic barrier did not differ significantly in thickness in the two abscesses but seemed moderately more necrotic on the cortisone side. It also invaded the surrounding tissues to a greater extent, probably because of the decrease in quantity and density of granulation tissue on this side. When, as occurred in certain small areas, the granulation tissue was completely absent, the invasion of the surrounding tissue was quite marked. The granulation tissue layer on the cortisone side differed from that of the control side in the following respects:

(a) It was much thinner—usually less than one-half the control and sometimes practically completely absent.

(b) The fibroblasts were smaller, flatter and less branched. They appeared more compact in some regions while in others they were quite widely separated by edema. They did not tend to align themselves in rows parallel to the young capillary beds, as did the controls.

(c) There was a greater profusion of histiocytes and lymphocytes as well as polymorphonuclear infiltration into the granulation tissue.

(d) Vascularization seemed to be as prominent as on the control side, but very young capillary buds were more sparse.

In order to study the possible effect of vasoconstriction or ischemia upon granulation tissue in comparison with the inhibitory effect of cortisone, the following experiments were performed: The left iliac artery and vein were ligated just below the bifurcation of the aorta in 5 rats. One-half cubic centimeter of turpentine was then injected subcutaneously into the thigh on the ligated side and the normal side as well. The ischemic leg remained viable and after 4 days the animals were sacrificed. Toluidin blue was injected into the abdominal aorta and the distribution of dye in both extremities was observed. In all instances, dye entered the normal side immediately and the entire limb became deeply stained. On the ligated side there was a gradual and only slight evidence of dye penetration. The abscesses were removed, fixed and examined as before. The iliac arteries of three other animals were ligated in the same fashion and daily skin temperatures were taken on each thigh with a Taylor Diatherm. The skin temperatures

---

11. Ingle, D. J., *J. Clin. Endocrinol.*, 1950, v10, 1312.

12. Fritz, I., and Levine, R., *Am. J. Physiol.*, in press.

13. Woodbury, D. M., and Sayers, G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 398.

14. Torda, C., and Wolff, H. G., *J. Pharm. and Exp. Ther.*, in press.

15. Taubenhaus, M., and Amromin, G. D., *Endocrinol.*, 1949, 44, 359.

showed a consistent difference between the ligated and the normal sides, in that the operated side was one to two degrees (C) cooler than the normal leg. Despite the undoubted restriction of blood supply on the operated side, no significant difference was found in the granulation tissue as compared to the control side, both presenting typical turpentine abscesses of 4 days' duration and showing intense leukocytic and good granulation response. No necrosis of the structures surrounding the abscess was observed.

To investigate the possible nervous mechanism we studied abscess formation in denervated areas. In 5 animals the left sciatic nerve was transsected and turpentine was injected subcutaneously into both thighs posteriorly, on the day of operation. Daily injections of 5 mg cortisone were given into the scapular regions for five days, the animals were then sacrificed, the abscesses removed and prepared as above. Three other animals were similarly operated on, and one week was permitted to elapse, to allow degeneration of nerve endings beyond the transsection. Abscesses were then induced and cortisone given as before. The abscesses on both the denervated and control sides exhibited typical cortisone effects, no appreciable difference being noted in either leukocytic barrier or qualitative and quantitative aspects of the granulation tissue layer.

To study the effect of a more radical and extensive denervation, spinal cord transection at the level of about T_8 was performed and the distal portion of the cord removed as much as possible. Twenty-four hours later the lumbar sympathetic fibers were removed. Turpentine was then placed subcutaneously in the suprascapular region, which was well supplied by nerves, and the lumbar region which was included in the paralyzed portions of the animal. Two animals received cortisone and 3 were kept as controls. Four-day-old abscesses were then studied, the abscess of the denervated part compared with the abscess of the intact site. The granulation tissue of these animals in general was poorer than normal, due to the general debilitated state of these rats following the operative procedure. The degree of inhibition however was far greater in the cortisone treated animals. No difference was noted in the intact and denervated parts of the animals. There was no interference with cortisone action in the denervated area.

Discussion. The local action of cortisone was demonstrated by mixing and injecting it with turpentine and showing typical inhibition of the granulation tissue in the developing abscess. This inhibition did not follow absorption of the cortisone from the site of injection with a general cortisone effect in the whole animal, for granulation tissue in a simultaneously developing abscess following turpentine injection (without cortisone) in another site in the same animal showed no inhibition. It is therefore clear that the changes seen in granulation tissue developing under cortisone influence do not depend on an altered metabolic state induced by cortisone in the whole animal, *e.g.* the catabolic action of cortisone on proteins in the whole animal is not responsible for its inhibitory effect on granulation tissue. In a previous paper(1) inhibition of granulation tissue proliferation following sex steroid administration was reported and evidence was presented that this might be due to inhibition of growth hormone output from the anterior pituitary by the steroid injected in these animals. Our experiments show that cortisone inhibition of granulation tissue could not occur by this mechanism, if so, both the cortisone and the control sides would have been inhibited in the same manner. The local action of cortisone has also been demonstrated by other methods in experimental animals(9) and by clinical observations on inflammatory conditions of the eye(16).

Although it has been shown that animals lacking C_{11} oxysteroids cannot make adequate adjustments in the dynamics of circulation (17), and that adrenal extracts potentiate the vasoconstrictive action of nor-adrenaline in splanchnic vessels(12), our experiments demonstrated that granulation tissue de-

---

16. Koff, R., Rome, S., Kasper, R., Commons, R. R., Button, R., and Starr, R., *J.A.M.A.*, 1950, v144, 1259.

17. Goldstein, M. S., Ramey, E. R., and Levine, R., *Am. J. Physiol.*, 1950, v163, 561.

veloped well in spite of inadequate blood supply. The inadequacy of the blood supply was proven by delayed and decreased entrance of dye into the extremity and by the persistent lowering of the skin temperature. The ischemic extremity, however, received some blood as evidenced histologically by the lack of necrosis. The limb may have been kept viable by collaterals from the pelvic arteries and skin vessels. In these experiments it was shown that granulation tissue developed well in spite of inadequate blood supply. This seems to rule out ischemia as a cause for inhibition of fibroblastic proliferation by cortisone.

In our experiments, in which the sciatic nerve was transsected, the action of cortisone was evident in the denervated as well as in the intact extremity. The damage induced to motor and sensory fibers by transsection of the sciatic nerve of the rat has not been adequately studied and the amount of sympathetic fibers destroyed by this procedure is unknown. It was thought advisable therefore to perform a more radical denervation to corroborate these results. In animals with cord transsection and lumbar sympathectomy granulation tissue in denervated areas was affected by cortisone to the same degree as in intact areas.

The mechanism of the inhibitory effect of cortisone upon granulation tissue remains unknown. As each of the possibilities discussed above is ruled out, it becomes more likely that the effect is direct and locally exerted; presumably upon the growing fibroblasts and/or the matrix in which these cells develop.

Summary. Granulation tissue developing around turpentine abscesses in rats is inhibited if cortisone is suspended in the turpentine. This effect is a local one, because in the same animal a simultaneously developing abscess caused by turpentine in which cholesterol is suspended showed no such inhibition. Moderate ischemia does not inhibit granulation tissue formation and presumably does not underlie the inhibitory action of cortisone upon granulation tissue. Denervation does not interfere with this effect of cortisone. The implication of these findings on the possibility of a direct cortisone action upon granulation tissues is discussed.

Acknowledgement is made to Dr. Rachmiel Levine for his suggestions and criticisms.

Received March 27, 1951. P.S.E.B.M., 1951, v76.

A Study of the Fibrous Components of the Vitreous Body of the Electron Microscope.* (18655)

A. GEDEON MATOLTSY, JEROME GROSS, AND ANTONIO GRIGNOLO.
(Introduced by Chester N. Frazier)
From the Departments of Dermatology and Medicine, Harvard Medical School, and Massachusetts General Hospital, the Department of Biology, Massachusetts Institute of Technology, and the Retina Foundation, Massachusetts Eye and Ear Infirmary.

The vitreous humor of the mammalian eye has been described by Robertson and Duke-Elder(1) as "a gel composed of a meshwork of elastic fibrillae suspended in a viscous fluid." Young(2) and Mörner(3) considered the fibrous residue of the vitreous humor to be collagenous since it dissolved in boiling water and gelled on cooling. Pirie, Schmidt and Waters(4) extracted ox vitreous body with saline, solubilized the fibrous residue with hot hydrochloric acid and studied this substance by means of X-ray diffraction, chromatography of the hydrolysate and swelling phenomena. The wide angle X-ray diffraction pattern of the stretched fibrous material was typical of the fiber diagram of the collagen class of proteins(5). Paper chromatography revealed glycine, proline, hydroxyproline, alanine, asparate and glutamate in amounts comparable to those found on the chromatographs of hydrolysed gelatin. From these data and the shrinkage and swelling properties they concluded that the vitreous body consists of a network of collagen-like fibrils imbedded in a jelly consisting of hyaluronic acid and protein. In a recent paper published during the course of the present investigation Schuchardt and Knoch(6) described a meshwork of thin ill-defined fibrils, in formalin fixed vitreous body, observed in the electron microscope.

---

* This work was supported in part by grants from the Rockefeller Foundation, the Veterans Administration, the American Optical Co., the U. S. Public Health Service, and the Robert W. Lovett Memorial Foundation.

1. Robertson, E. B., and Duke-Elder, W. S., *Proc. Roy. Soc. Lond. B.*, 1933, v112, 215.
2. Young, R. A., *J. Physiol.*, 1894, v16, 325
3. Mörner, C. T., *Z. f. Physiol. Chem.*, 1894, v18, 233.
4. Pirie, A., Schmidt, G., and Waters, J. W., *Brit. J. Ophthal.*, 1948, v32, 321.

This paper describes the results of a preliminary study with the electron microscope of the fibrous components of the vitreous body.

Methods. Cattle eyes obtained 3-6 hours after death were used. The vitreous humor was removed intact from the eye and the adhering tissue fragments were carefully dissected away. Fifteen to 20 vitreous bodies were fragmented in the Waring blendor for 5-10 minutes. Foaming occurred during this period, but subsided spontaneously within 20-30 minutes. The resulting fluid was quite viscous. A small amount of sediment produced by centrifugation at 3,000 r.p.m. for 20 minutes showed large fibers, broken membranes and granular debris in the light microscope. The water clear supernatant was then centrifuged at 18,000 r.p.m. for 30-40 minutes and a white, sticky sediment was obtained, which when dried accounted for 0.0028% of the fresh vitreous body by weight. The sediment was then resuspended in distilled water. Similar preparations were made from the central portion of the vitreous body and from the peripheral regions. The periphery was arbitrarily defined as the outer third.

All operations were performed at temperatures of 0-5°C. The above-described high-speed sediments were prepared for electron microscopy by placing a drop of the well-stirred water suspension on the collodion-supporting film of the specimen grid. The preparations were drained and shadowed with 6-8 mg of chromium at 20 cm filament to specimen distance at angles of 6, 8 or 10 degrees. The suspensions were necessarily diluted with distilled water to reduce the amount of material deposited on the film. Some preparations were stained with a 1.0% solution of phosphotungstic acid and were examined directly or shadowed in addition. PTA tends to clean up the background considerably. An RCA type EMU electron microscope in the Department of Biology, Massachusetts Institute of Technology, was used for these studies.

Results. Electron micrographs of aqueous suspensions of fragmented whole bovine vitreous sediment revealed three distinct types of fibrils and a moderate amount of a fine amorphous material. The most abundant form was represented by a long thin unbranched fibril, uniform in width and curving

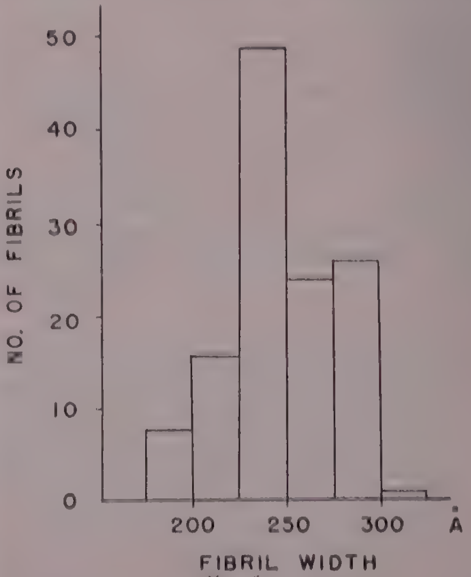


Fig. 1.

Distribution of fibril widths in Angstrom units for smooth fibrils.

---

5. Astbury, W. T., *J. Int. Soc. Leather Trades Chem.*, 1940, v24, 69.

6. Schuchardt, E., and Knoch, M., *Naturwissenshaften*, 1950, v18, 426.

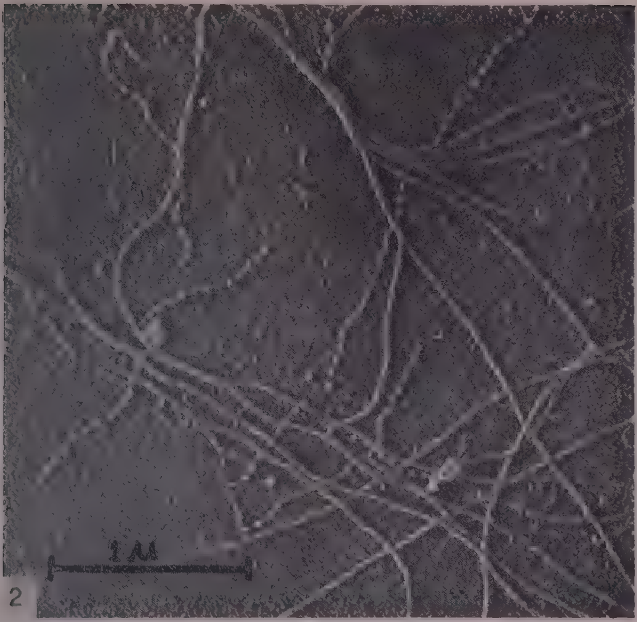


Fig. 2.
Electron micrograph of fragmented diluted sediment of fresh cattle vitreous showing two distinct fibril forms. Chromium shadowed. 29000×.

in relatively smooth arcs. Fibril widths ranged from 150-300 Å, averaging 250 Å (Fig. 1). Occasionally flattened fibrils were also observed which often frayed into extremely thin filaments. There was some suggestion of a very fine axial repeating period in some micrographs, but this was not resolved adequately or consistently enough to warrant determination of period at this time (Fig. 2).

The second type of fibril present in moderate number as compared to that described above, exhibited wide dispersion of length, was more tortuously bent and was clearly striated. The width of the fibrils averaged about 150-200 Å. No branching or fraying was observed. The axial periodicity varied between 500-850 Å, averaging 610 Å (Fig. 3). Resolution has not been adequate to reveal more than a suggestion of fine structure within the axial repeating period. The relative number of these thin striated fibrils varied greatly from preparation to preparation and in general were much less numerous than the non-striated form.

In addition to the new fibrous type described above, small numbers of striated fibrils about 500-800 Å in width, characteristic of collagen, have been seen in most preparations.

Examinations of sediments prepared from the peripheral and central parts of the vitreous body revealed all three types of fibrils to be present in each instance.

Discussion. The presence of a fibrous structural element in the vitreous body was suspected at least as far back as 1894 and was termed "residual protein" at that time by Mörner. Examination of the fresh vitreous body by light microscopy in the living state and after removal from the eye revealed the presence of membranes and fibers(7). In this study membranes were only observed in the low-speed sediment by light microscopy. Schwarz and Schuchardt(8) and Grignolo (unpublished), using the phase contrast micro-

---

7. Duke-Elder, W. S., *Br. J. Ophthal.*, 1930, Suppl. 4.

8. Schwarz, W., and Schuchardt, E., *Z. f. Zellforsch. u Mikr. Anat.*, 1950, v35, 292.

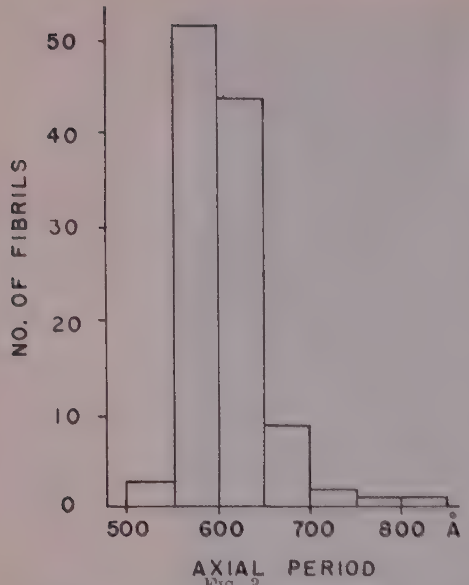


FIG. 3.

Distribution of axial period in Angstrom units for headed fibrils.

scope, observed large fiber bundles which frayed into finer fibrils in the fresh vitreous body. Whether or not these large fibers represent bundles of the finer elements observed in the electron microscope will remain uncertain until they can be examined directly after microdissection.

There is no information at this time as to whether the three fibrous elements are uniformly distributed throughout the vitreous body or are individually localized to specific regions or structures.

It is reasonable to presume that the small fraction of collagen fibrils is responsible for the wide angle collagen pattern obtained by Pirie *et al.*(4). However, it is also quite possible that the other two fibrous units may belong to the collagen class of proteins and also contribute to the X-ray diagram. The periodicity of the fine, striated fibrils is sufficiently close to 640 Å, characteristic of collagen, to suggest a close relationship to collagen.

However, Krause(9) and Schaeffer and Murray(10) have reported significant amounts of tryptophane in vitreous hydrolysates, an amino acid characteristically absent from collagen, suggesting the presence of non-collagenous protein.

By checking the morphology of the elements separated from the vitreous body with the electron microscope it is hoped to produce homogeneous fractions of the different structural elements thereby permitting more definitive chemical analyses.

Summary. 1. Electron micrographs have been obtained of a fibrous fraction separated from fragmented fresh vitreous humor of cattle eyes by centrifugation, which reveal the presence of three morphologically distinct fibril types. 2. The least numerous species is represented by a fibril 500-800 Å in width having an axial periodicity of about 640 Å and intraperiod structure characteristic of collagen. 3. A second fibrous component measuring 150-200 Å in width is characterized by a repeating axial period averaging about 610 Å. 4. A third and most abundant element is a fibril averaging 250 Å in width exhibiting only a suggestion of a very fine axial repeating period.

---

9. Krause, A. C., *Biochemistry of the Eye*, 1934, The Johns Hopkins Press, Baltimore.

10. Schaeffer, A. J., and Murray, J. D., *Arch. Ophthal.*, 1950, v44, 833.

Received April 5, 1951. P.S.E.B.M., 1951, v76.

Anaerobic Glycolysis in Fortified Cell-free Homogenates of Tapeworm Tissue.* (18656)

Clark P. Read.[†] (Introduced by F. Crescitelli)
From the Biology Department, Rice Institute, Houston, Texas.

There is evidence that the anaerobic degradation of carbohydrate in the tapeworm, *Hymenolepis diminuta,* occurs by processes involving the transfer of phosphate radicals (1). The mechanisms are probably similar to those occurring in vertebrate animals.

The present communication is concerned with a study of the glycolytic activity of fortified cell-free preparations of tapeworm tissues. LePage(2) described a cell-free system in which he was able to maintain glycolysis and an over-all esterification of inorganic phosphate using mammalian tumor homogenates as the source of enzymes. In the present study LePage's system was used as a basis, and the individual ingredients of the system were varied in an attempt to determine optimal conditions for lactate production and phosphate esterification.

Procedure. The tapeworm, *Hymenolepis diminuta,* was maintained in the laboratory in albino rats(1). The following procedure was used in all experiments: Reaction vessels were prepared before the tissue was homogenized. All reactants were added except the homogenate, and the vessels kept in cracked ice until the addition of the homogenate. The basic system contained .0024 M K_2HPO_4, .025 M $KHCO_3$, .040 M nicotinamide, .00033 M adenosine triphosphate (A.T.P.), .00020 M diphosphopyridine nucleotide (D.P.N.), .002 M hexose diphosphate, .005 M pyruvate, .0066 M $MgCl_2$, .010 M KF, .010 M glucose, and .2 ml of 25% homogenate. The total volume of the reaction mixture was 3 ml. The worm tissue was homogenized in a chilled all-glass homogenizer after the addition of 3 parts (by weight) of ice-cold .154 M KCl (made alkaline by adding 8 ml of .02 M $KHCO_3$ per liter). Preliminary experiments using water homogenates yielded highly irregular results. The use of alkaline KCl in homogenizing greatly improved reproducibility. Completeness of homogenization was checked microscopically and in all preparations at least 90% of the cells were broken. The pH of the reaction mixtures in all experiments was 7.4. After addition of the homogenate the vessels were gassed with pure nitrogen and incubated at 38°C for 30 minutes. The reaction was terminated by the addition of .2 ml of 100% trichloracetic acid, with mixing, to each vessel. After neutralization with strong KOH (to pH 8.2) and rapid centrifugation, aliquots of the medium were analyzed for lactic acid(3) and inorganic phosphate (4). In all experiments unincubated reaction mixtures were similarly analyzed. From these data it was possible to calculate the lactate formed and phosphate esterified under the varying conditions. The results obtained in these experiments are summarized in Table I.

In attempting to find an optimal system for sustaining phosphate bond energy and lactate production some compromise is necessary since several enzymes are concerned in the reactions. Addition of fluoride to the medium probably blocks the reactions at the stage of phosphoglyceric acid and limits phosphatase activity. Addition of fluoride causes accumulation of phosphoglyceric acid in tapeworm tissue extracts to which H.D.P. has been added(1) and this tapeworm possesses a fluoride-sensitive, magnesium-activated A.T.P.-ase(5). Because of fluoride-blocking, pyruvate was added as hydrogen acceptor. However, the evidence that pyruvate functions

---

* Supported in part by a grant from the U. S. Atomic Energy Commission.

[†] National Research Council—Atomic Energy Commission Fellow, Biological Sciences. Present address: Department of Zoology, University of California, Los Angeles.

1. Read, C. P., *Exp. Parasitol.,* 1951, in press.

2. LePage, G. A., *J. Biol. Chem.,* 1948, v176, 1009.

3. Barker, J. B., and Summerson, W. H., *J. Biol. Chem.,* 1941, v138, 535.

4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375.

5. Read, C. P., *J. Parasitol. Suppl.,* 1949, v35, 26.

TABLE I. Effect of Varying Concentration of Cofactors on Lactate Production and Phosphate Esterification by Tapeworm Homogenates.

Exp.	Component varied	P esterified, µM	Lactate formed, µM
	Pyruvate		
1	.005 M	+2.33	1.52
	0	—1.74	0.34
2	.005	+2.06	1.61
	0	—1.48	0.24
	Hexose diphosphate		
3	.002 M	+1.81	1.95
	.001	+1.52	1.55
	0	+1.20	0.88
4	.002	+2.01	1.69
	.001	+1.65	1.12
	0	+1.08	0.64
	$MgCl_2$		
5	.0033 M	+2.39	1.24
	.0066	+1.94	1.73
	.0099	+1.84	0.95
6	.0033	+2.09	1.19
	.0066	+1.89	1.80
	.0099	+1.41	0.71
	A.T.P.		
7	.0002 M	+3.75	1.24
	.0004	+4.48	2.03
	.0008	+2.52	1.55
8	.0002	+3.53	1.49
	.0004	+4.63	2.14
	.0008	+2.07	1.78
	D.P.N.		
9	.0002 M	—0.38	0.81
	.0004	+1.62	1.67
	.0006	+3.81	1.98
10	.0002	—0.68	0.24
	.0004	+1.87	1.79
	.0006	+3.90	2.13
	Nicotinamide		
11	.02 M	+1.42	1.36
	.04	+2.16	2.27
	.08	+3.58	2.61
12	.02	+1.31	1.14
	.04	+2.02	1.88
	.08	+3.44	2.89
	Inorganic P		
13	.0010 M	+0.11	0
	.0015	+1.41	0.65
	.0025	+2.94	1.78
14	.0010	+0.10	0.09
	.0015	+1.07	1.12
	.0025	+2.61	1.94
	KF		
15	.0066 M	+2.97	0.95
	.0100	+1.62	2.02
	.0200	+1.00	1.61
16	.0066	+2.48	0.65
	.0100	+1.95	1.87
	.0200	+1.09	1.30

as a hydrogen acceptor in the present experiments is only presumptive. When A.T.P. or hexose diphosphate were not added to the reaction mixture a negligible amount of lactic acid was produced and the amount of inorganic phosphate esterified was significantly lower. The lower net uptake of inorganic phosphate at higher concentrations of A.T.P. is probably due to increased phosphatase activity. However, it is difficult to account for the lowered lactate production with higher A.T.P. concentrations. The decrease in inorganic phosphate esterified at higher concentrations of magnesium is probably due to phosphatase activation(5), but again the lowered lactate production under these conditions is peculiar. It would be of interest to determine whether calcium ion which antagonizes magnesium activation of phosphatase in this animal(5) would have an effect on the glycolytic activity of the present system.

Obviously, the optimum concentration of D.P.N. was not determined in these experiments, but it is also obvious that D.P.N. is necessary for phosphate esterification and lactate production. Nicotinamide probably functions in protecting D.P.N. from the activity of enzymes which destroy it(2).

These experiments may furnish additional evidence that the reactions, glucose→lactate, proceed by processes similar to those in vertebrate tissues. This is not meant to imply that lactate is the only product of glycolysis in tapeworms. This work indicates that anaerobic glycolysis can be maintained in a cell-free system, with the tissues of this tapeworm as the source of enzymes.

LePage's system which is optimal for mammalian tumor tissues is not optimal for worm tissue homogenates. The data indicate that a more nearly optimal system contains the following: .005 M pyruvate, .002 M hexose diphosphate, .0033 M $MgCl_2$, .0004 M A.T.P., .0006 M D.P.N., .08 M nicotinamide, .0025 M phosphate, .0066 M KF, .025 M $KHCO_3$, .010 M glucose, and the homogenate. Further study of the system will be necessary to establish that these conditions are actually optimal in all respects.

Summary. Lactate production and phosphate esterification were followed in dilute cell-free homogenates of the rat tapeworm,

Hymenolepis diminuta. The system was fortified with bicarbonate, magnesium chloride, nicotinamide, diphosphopyridine nucleotide, adenosine triphosphate, hexose diphosphate, pyruvate, and glucose. Fluoride was used to inhibit phosphatase activity. Semi-optimal conditions were determined. The data support the idea that glycolysis in tapeworms proceeds by processes similar to that in vertebrate tissues.

Received March 15, 1951. P.S.E.B.M., 1951, v76.

AUTHORS' INDEX

VOLUME 76

(*The numerals indicate the page*)

Prepared by Dr. William Antopol

Abrams, H. L. 729.

Adams, C. 292.

Adams, E., and White, A. Vitamin C adrenal. 322.

Aikawa, J. K., Knight, V. H., and Tyor, M. P. Digitoxin, effect. 250.

Albaum, H. G., Hirschfeld, A. I., Tonhazy, N. E., and Umbreit, W. W. Hormone, cortisone, phosphocreatine and adenosine triphosphate. 546, 247.

Alex, M. 714.

Allen, T. H. Dye, T-1824, extraction. 145.

Almquist, H. J., and Johnson, C. Antibiotics, hexamitiasis effect. 522.

Alonso, D. 777.

Ambrose, A. M. Antibiotic, streptomycin toxicity. 466.

Ames, R. G., and Van Dyke, H. B. Hormone, antidiuretic, inactivation. 576.

Amrein, Y. U. Antibiotic tyrocidine, trypanosome. 744.

Ancowitz, A. 21.

Anderson, C. E. 216.

Anderson, G. E., Wiesel, L. L., Hillman, R. W., and Stumpe, W. M. Hormone ACTH, sulfhydryl inhibition. 825.

Anderson, R. C. 372.

Andrew, V. W. 79.

Arhelger, S. W., and Huseby, R. A. Hormone estrogen, androgen antagonism. 811.

Armstrong, W. D. 229.

Artom, C. 552.

Axelrod, A. E. 665, 670.

Babcock, G. M. 382.

Bacchus, H. Heart hypertrophy, and KCl. 391.

Back, A. 105.

Bahner, C. T. 278.

Baker, B. L. 353.

Baker, H. W., Anderson, C. E., McCluer, R. H. Bromsulphthalein, excretion. 216.

Baker, J. H. 254.

Bakwin, H. 756.

Balla, G. A. 496.

Barbee, K. W., and Connor, Johnson B. Vitamin B_{12}, metabolism. 720.

Basinski, D. H. 704.

Batchelder, T. L. 375.

Bateman, J. H. 65.

Bauer, R. O. 767, 847.

Bayles, T. B. 274.

Beatty, J. O. 222.

Becker, H. C., Hodes, R., Founds, W. L., Jr., and Peacock, W. M., Jr. Physiological recording. 761.

Bell, M. C., Whitehair, C. K., and Gallup, W. D. Antibiotic, aureomycin, digestion effect. 284.

Berger, E. Y., Quinn, G. P., and Homer, M. A. Hormone DCA, colon. 601.

Berlin, N. I., Hyde, G. M., Parsons, R. J., Lawrence, J. H., and Port, S. Blood volume, method. 882, 831.

Bernheim, F. 133, 338.

Bern, H. A. 499.

Bernthal, T., Greene, W., Jr., and Revzin, A. M. Heart, acceleration, chemoreceptors. 121.

Beyer, E. 267.

Bien, E. J., Ziff, M., and Bunim, J. J. Muscle, chemistry, rheumatism. 649

Bigelow, R. R., Furth, J., Woods, M. C., and Storey, W. H. X-radiation, endothelial damage. 734.

Birkaug, K. Vaccine BCG, viability. 311.

Birkeland, J. M. 467.

Black, M. M., and Kleiner, I. S. Succinate, tumor metabolism. 437.

Black, M. M. 446.

Bloch-Frankenthal, L., and Back, A. Colchicine, tumor growth. 105.

Bloom, W. L., and Willcox, M. L. Dextran in blood and urine. 3.

Boger, W. P., Matteucci, W. V., and Beatty, J. O. Benemid, effect on antibiotics. 222.

Bohnhoff, M. 679.

Booth, A. N. 540.

Borison, H. L., and Wang, S. C. Glycosides action, emetic. 335.

Bosch, J. K. 777.

Bothwell, J. 38.

Bowman, R. H. Ova, fertilization. 129.

Boyd, L. J. 10.

Brahin, C. M. 86.

Braun, W. 786.

Brecher, G. 396, 456.

Breslin, D. 242.

Briggs, G. M. 417.

Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H. Vit. B_{12}, assay. 806.

Brown, R. 93.

Brown, R. W., Woodbury, D. M., and Sayers, G. Hormone, prolactin, phosphorus metabolism. 639.

Brucer, M. H. 278.

Brueck, J. W., and Buddingh, G. J. Fungi, cultivation, yolk sac. 258.

Buddingh, G. J. 258.

Buehler, H. J., Katzman, P. A., and Doisy, E. A. Enzyme glucuronidase, *E. coli*. 672.

Bunim, J. J. 649.

Burch, G. E. 1.

Burchenal, J. H., and Babcock, G. M. Vitamin, citrovorum factor, Methopterin competition. 382.

Burgison, R. M. 759.

Burke, R. C. Coccidioides, parasitic phase cultivation. 332.

Burns, L. 598.

Burr, M. 330.

Busch, H. 38.

Cabell, C. A., and Earle, I. P. Protein, iodinated, protein depletion. 371
Callison, E. C., and Orent-Keiles, E. Vitamin E, eye abnormality. 295.
Calvin, L. B. 480.
Cameron, W. 267.
Cantarow, A. 432.
Cardullo, H. M., and Holt, L. E., Jr. Taste, phenyl thio carbamide, genetics. 589.
Carr, C. J., Burgison, R. M., Fassell, M. S., and Krantz, J. C., Jr. Nitrates, depressor response. 759.
Carter, B. B. 665, 670.
Cass, W. A. 493.
Castor, C. W., Baker, B. L., Ingle, D. J., and Li, C. H. ACTH effect, brain. 353.
Cayer, D. 552
Cerecedo, L. R. 497
Chaikoff, I. L. 563
Chambers, F. W. 282.
Chambers, V. C., Smith, M. W., Evans, C. A. Virus, neurotropic, in testis. 213.
Chambers, V. C. 696.
Chapman, W. H. 456, 282, 396.
Chase, H. B., and Monteagna, W. Hair proliferation and skin damage. 35.
Cheever, F. S. 571
Chen, K. K., Anderson, R. C., and Henderson, F. G. Bufalin, digitalis effect. 372.
Chen, K. K. 530.
Cheng, D. W. 24.
Chow, B. F., Rosenblum, C., Silber, R. H., Woodbury, D. T., Yamamoto, R., and Lang, C. A. Isotope cobalt, vitamin B_{12}, metabolism. 593.
Christie, J. H. 207.
Chu, T. H., Cheever, F. S., Coons, A. H., and Daniels, J. B. Virus, mumps, distribution in monkey. 571.
Clark, E. M. 379.
Co, Tui. 226.
Cochran, K. W. 422.
Cohen, S. L., and Huseby, R. A. Enzyme glycuronidase, serum. 304.
Cole, V. V. 62
Combs, G. F. 46.
Comroe, J. H., Jr. 725.
Constantinides, P. 77.
Coon, J. M. 543.
Coons, A. H. 571.
Cooperman, J. M., Rubin, S. H., and Tabenkin, B. Niacin in Borrelidin toxicity. 18.
Cornatzer, W. E., Artom, C., Herrell, G. T., Jr., and Cayer, D. Vit. B_{12}, P^{32} toxicity. 552.
Couch, J. R. 58, 265.
Coulson, R. A. 175.
Cronkite, E. P., Brecher, G., and Chapman, W. H. X-ray, glutathione protection. 396.
Cronkite, E. P., Chapman, W. H., and Brecher, G. Glutathione, irradiation, protection. 456.
Cronkite, E. P., Chapman, W. H., and Chambers, F. W. Radiation, flavonoid effect. 382.
Cross, J. B. Diasone, in toxoplasmosis. 748.
Cunha, T. J. 173.
Cury, A. 341.

Daniels, J. B. 571.
Davidow, B., and Frawley, J. P. Benzene hexachloride, metabolism. 780.
De Bodo, R. C., Schwartz, I. L., Greenburg, J., Kurtz, M., Earle, D. P., Jr., and Farber, S. J. Hormone, growth, water metabolism. 612.
De Bodo, R. C. 21, 608.
De Eds, F. 540.
Dellamonica, E. S. 193.
Dennis, C. 737.
De Robertis, E. 515.
De Venanzi, F. Hormone ACTH effect, serum phosphorus. 770.
Dieke, S. H. Thiourea, gastric evacuation. 788.
Doisy, E. A. 672.
Doolan, P. D. 418.
Dordoni, F. 559.
Dorfman, R. I. 203.
Dornbush, A. C., Oleson, J. J., Whitehill, A. L., and Hutchings, B. L. Antibiotic aureomycin, alkali treated, activity. 676.
Dorrance, W. R. 763.
Doubilet, H., and Mulholland, J. H Pancreas, duct, intubation. 113.
Doud, T. 748
Dougherty, T. F. 460.
Doull, J. 422.
Drabkin, D. L. Hemin chromoprotein biosynthesis. 527.
Drury, F. H., Albaum, H. G., and Hirshfeld, A. Adenosine triphosphate solubility. 247.
Dubois, K. P., Cochran, K. W., and Doull, J. X-ray, citrate synthesis. 422.
Dubois, K. P. 427.
DuBois, R. 292.
Ducommun, P., Timiras, P. S., and Dordoni, F. Hormones, effect, hemoglobin spreading. 559.
Duffy, C. E. Virus encephalitis Japanese B, rats. 566.
Dworecki, I. J. 10.

Earle, D. P., Bakwin, H., and Hirsch, D. Blood, potassium, newborn. 756.
Earle, D. P., Jr., De Bodo, R. C., Schwartz, I. L., Farber, S. J., Kurtz, M., and Greenberg, J. Hypophysectomy, electrolyte and water metabolism 608.
Earle, D. P., Jr. 612.
Earle, I. P. 371
Eckenhoff, J. E., and Comroe, J. H., Jr. Lobelin, tetraethyl ammonium effect. 725.
Edwards, H. M., Cunha, T. J., Meadows, G. B., Shawver, C. B., and Pearson, A. M. Vitamin APF, multiple factors. 173.
Eisenberg, E. 406.
Ellison, R. R., Wolfe, S., Lichtman, H., Ginsberg, V., and Watson, J. Vitamin, citrovorum factor, pernicious anemia. 366.
Elminger, P. J. 707.
Elvehjem, C. A. 441.
Emmett, J. W. 65.
Enselberg, C. D. 797.
Evans, C. A. 213, 696.
Ezell, H. K. 727.

Falk, D. 110.
Farah, A., Mook, W., and Johnson, R. Mercury, heart. 403.

Farber, M. 408.
Farber, S. J. 608, 612.
Fassell, M. S. 759.
Favour, C. B. 274.
Feigelson, P. 441.
Feinstein, R. N. Radiation, , nucleoprotein viscosity. 646.
Ferreira-Berrutti, P. Nerve, deflection, experimental. 302.
Figge, F. H. J. 210.
Finerty, J. C., and Panos, T. C. Parabiosis intoxication. 833.
Fink, K. Urine, ninhydrin reducing. 692.
Finland, M. 560.
Fitzpatrick, T. B. 346.
Flesch, P. Squalene, hair loss. 801.
Follis, R. H., Jr. Enzyme, proteolytic, collagen metachromasia. 272, 722.
Formaggio, T. G. Blood factor O, newborn. 554.
Fortier, C., Skelton, F. R., and Constantinides, P. Adrenal function and blood sugar. 77.
Founds, W. L., Jr. 761.
Fowler, J. C. 793.
Franchi, C. M., and DeRobertis, E. Tissue elastic, electron microscopy. 515.
Frankel, S. 289.
Franklin, A. E. 183.
Frawley, J. P. 780.
Freedman, R. I. 238, 239.
Freund, H. A. Virus effect. 704.
Freund, J. 717.
Friedell, H. L., and Christie, J. H. Isotope phosphorus effect on rat. 207.
Fry, F. J. 361.
Fry, W. J. 361.
Furth, J. 734.

Gabrilove, J. L. 763.
Gallup, W. D. 284.
Garcia, J. F., Van Dyke, D. C., Huff, R. L., Elmlinger, P. J., and Oda, J. M. Blood, erythrocyte volume, ACTH effect. 707.
Gard, S., and Heller, L. Virus Col-MM, hemagglutination. 68.
Gebhardt, L. P. 460.
Gedigk, P. 838.
Georgi, C. E., Militzer, W., Burns, L., and Heotis, J. Bacterial thermophilic, cell granule. 598.
Germuth, F. G., Jr., Nedzel, G. A., Ottinger, B., and Oyama, J. Hormone cortisone, anatomic changes. 177.
Gibson, D. M. 585.
Gigee, W. 97.
Gillen, A. 330.
Ginsberg, V. 366.
Glass, G. B. Jerzy, Boyd, L. J., and Dworecki, I. J. Protein, thermal coagulation. 10.
Glauser, K. F. 257.
Glick, C. 267.
Goddard, J. W. 145.
Goldberg, R. C., and Chaikoff, I. L. Thyroid, tumor after radioiodine. 563.
Goldblatt, H. 124.
Goldie, H., Calvin, L. B., Nash, H., and Hahn, P. F. Isotope, gold, macrophages. 480.
Goldie, H., Watkins, F. B., Powell, C., and Hahn, P. F. Isotope, gold, peritoneal fluid. 477.
Goldsmith, Y. 430.
Gollan, F., and Visscher, M. B. Brain, analysis, encephalomyelitis. 746.
Good, R. A. 190, 604.
Goodlow, R. J., Braun, W., and Mika, L. A. Bacteria brucella, resistance and virulence. 786.
Goodman, J. H. 65.
Gordon, G. S. 406.
Gorzynski, G. A. 493.
Gottshall, R. Y., Roberts, J. M., Portwood, L. M., and Jennings, J. C. Antibiotic, synnematin. 307.
Gourzis, J., and Bauer, R. O. Veratrum viride, toxicity. 767.
Grau, C. R. 741.
Gray, C. T. 467.
Green, M. N., Heath, E. C., and Yall, I. Furacin and sulfhydryl enzymes. 152
Green, M. 384.
Greenberg, D. 226.
Greenberg, J. 608, 612, 700.
Greenberg, L. D., and Rinehart, J. F. Cholesterol, plasma, and B_6 deficiency. 580.
Greene, W., Jr. 121.
Gregory, F. J. 286.
Grignolo, A. 857.
Gross, J., and Leblond, C. P. Thyroxine, metabolites. 686.
Gross, J. 857.
Gross, L. Leukemia, spontaneous, transmission. 27.
Grunert, R. R., and Phillips, P. H. Diabetes, uric acid. 642.
Gutierrez, J. 15.
Gyorgy, P., and Goldblatt, H. Liver necrosis, yeast effect. 124.

Habel, K., and Li, C. P. Virus, poliomyelitis, intraspinal inoculation. 357.
Hahn, F. E., and Wisseman, C. L., Jr. Antibiotic, enzyme inhibition. 533.
Hahn, P. F. 477, 480.
Haley, T. J., and Stolarsky, F. Enzyme histamine anti, plasma coagulation. 299.
Halick, J. V., and Couch, J. R. Antibiotics, fowl nutrition. 58.
Hall, F. G., and Hall, K. D. Consciousness time, altitude. 140.
Hall, K. D. 140.
Handler, P., and Bernheim, F. Kidney decapsulation, hypertension. 338.
Harrell, G. T., Jr. 552.
Harris, P. N. 530.
Hartsell, S. E. 455.
Hayes, S. P., Dougherty, T. F., and Gebhardt, L. P. Antibody, tissue, method. 460.
Hazan, E. L., and Brown, R. Antibiotic, fungicidin. 93.
Heath, E. C. 152.
Heidelberger, C., and LePage, G. A. Isotope, glycine, nucleic acid metabolism. 464.
Heller, L. 68.
Henderson, F. G., Harris, P. N., and Chen, K. K. Longilobine, liver effect. 530.

Henderson, F. G. 372.

Henstell, H. H., and Freedman, R. I. Enzyme hyaluronidase anti, serum. 238, 239.

Heotis, J. 598.

Hernandez, T., and Coulson, R. A. Iguana, biochemical studies. 175.

Hess, W. C., Kyle, L. H., and Doolan, P. D. Blood, glutathione after cortisone and ACTH. 418.

Hess, W. C. 783.

Hill, E. B., and Briggs, G. M. Vitamin, pteroylglutamic acid replacement folinic acid. 417.

Hillman, R. W. 825.

Hinckley, G. V. 65.

Hirsch, D. 756.

Hirschfeld, A. 247.

Hirshfeld, A. I. 546.

Hodes, R. 761.

Hogan, A. G. 349.

Hollander, F. 49, 578.

Holman, R. T. Acid fatty, essential, metabolism. 100.

Holt, L. E., Jr. 589.

Holt, M. W., and Warren, S. Isotope I 131 radioautograph. 4, 709.

Holze, W. A. 325.

Homer, M. A. 601.

Horsfall, F. L., Jr. 116.

Horvath, S. M. 410.

Hoyt, H. S., Doud, T., and Hoyt, R. S. Testosterone, anabolic effect. 748.

Hoyt, R. S. 748.

Huff, R. L. 707.

Hulpieu, H. R., and Cole, V. V. Procaine, antidotes. 62.

Humphreys, E. M. 234.

Hurley, P. D. 804.

Huseby, R. A. 304, 811.

Hussey, C. V. 732.

Hutchings, B. L. 676.

Hyde, G. M. 831.

Ingle, D. J. 353.

Jackson, C. 578.

Jackson, N. R. 65.

Jaffe, B. 267.

Janowitz, H. D., and Hollander, F. Vagus, gastrin. 49.

Janowitz, H. D., Hollander, R., and Jackson, C. Mucus, gastric, acetylcholine stimulation. 58.

Jaques, R. 585.

Jennings, J. C. 307.

Jerzy Glass, G. B., Pugh, B. L., and Wolf, S. Stomach, mucus, neutralizing property. 398.

Johnson, B. Connor. 720.

Johnson, C. 522.

Johnson, R. 403.

Josephson, E. S., Taylor, D. J , Greenberg, J., and Ray, A. P. Pamaquine, metabolite. 700.

Juhn, M. Hormone, growth, feather regeneration. 118.

Jukes, T. H. 73, 806.

Kahler, H. 656.

Kahn, R. H. 499.

Kalter, S. S. Virus, influenza, inapparent infection. 570.

Kammandel, H. C. 170.

Kaplan, A. S., and Melnick, J. L. Virus Coxsackie, oral administration. 312.

Karnofsky, D. A. 289.

Kass, E. H., Neva, F. A., and Finland, M. ACTH, virus toxicity. 560.

Katzman, P. A. 672.

Kay, J. H., Balla, G. A., and Lamb, W. Histamine, anti, peritonitis. 496.

Kelley, B., Northrup, M., and Hurley, P. D. Vitamin, riboflavin, leucocyte production. 804.

Kiang, S. P. 21.

Kibrick, A. C., Palmer, D., and Skupp, S. Calcium, determination. 115.

Kilbourne, E. D., and Horsfall, F. L., Jr. Viritis, cortisone effect. 116.

King, E. R. 278.

Kirsner, J. B. 159.

Kittle, C. F. Batchelder, T. L., and Schafer, P. W. Histamine, effect, after gastrectomy. 375.

Kittle, C. F., King, E. R., Bahner, C. T., and Brucer, M. H. Isotope hafnium metabolism. 278.

Kleiner, I. S. 437.

Kline, I. T., and Dorfman, R. I. Citrovorum factor and oviduct response to stilbestrol. 203.

Knight, V. H. 250.

Knowles, W. B. Brain electrode, implanted. 315.

Koffler, H., and Markert, I. L. Nucleic acid, photodynamic action. 90.

Kolarsick, A. J. 170.

Koler, R. D., and Mann, J. D. Lymph, iron content. 221.

Korey, S. R. Dilantin effect, axon. 297.

Krantz, J. C., Jr. 759.

Kraus, S. D., Weintraub, S., and Wright, L. T. Metamine tri-ethylene, action. 489.

Kring, J. P., and Williams, J. N., Jr. Enzyme, xanthine oxidase, liver. 828.

Kritchevsky, D., Grau, C. R., Tolbert, B. M., and Krueckel, B. J. Acetate sodium, distribution in egg. 741.

Krop, S. 538.

Krueckel, B. J. 741.

Kunkel, H. G., Slater, R. J., and Good, R. A. Myeloma, protein and gamma globulin. 190.

Kupfer, S. Heart, coronary artery occlusion, shock. 134.

Kurtz, M., deBodo, R. C., Kiang, S. P., and Ancowitz, A. Hypoglycemia, growth hormone. 21.

Kurtz, M. 608, 612.

Kyle, L. H. 418.

Lamb, W. 496.

Landis, L., and Krop, S. Histamine effect, on fungistasis of antihistaminics. 538.

Lang, C. A. 393.

Lansing, A. I., Roberts, E., Ramasarma, G. B., Rosenthal, T. B., and Alex, A. Artery, elastin, amino acid content. 714.

Laskowski, L., Stanton, M. F., and Pinkerton, H. Alloxan, therapy Bartonellosis. 475.

Lawrence, J. H. 831.

Layton, L. L. Hormone cortisone, chondroitin sulfate synthesis. 596.

Leary, D. E. 738.
Leblond, C. P. 686.
Lee, R. E., and Holze, W. A. Vitamin C, shock. 325.
LePage, G. A. 464.
Levin, J. K. 435.
Levine, M. Leprosy, tuberculin agglutinins. 171.
Li, C. H. 353, 836.
Li, C. P. 357.
Lichtman, H. 366.
Longfellow, D. 435.
Losee, F. L. Neidig, B. A., and Hess, W. C. Acid, amino, dentin protein. 783.
Lown, B., Saltzberg, H. Enselberg, C. D., and Weston, R. E. Potassium metabolism, digitalis toxicity. 797.
Lowry, J. S. 773.
Ludovici, P. P., Axelrod, A. E., and Carter, B. B. Antibody, pantothenic acid deficiency, methionine and pyridoxine effect. 665, 670.
Lusky, L. M. 765.

Mack, H. P., and Figge, F. H. J. Antibody, hemagglutinin, hetero. 210.
Mackler, B. 574.
Maisel, B. Peritoneum, pneumo, gastrectomy preparation. 484.
Maison, G. L. 486.
Mann, R. D. 221.
Markert, I. L. 90.
Marshall, H. C. 159.
Martin, C. 110.
Matoltsy, A. G., Gross, J., and Grignolo, A. Eye, vitreous, electron microscopy. 857.
Matteucci, W. V. 222.
Mayer, J. Goddard J. W. Gonadotropin, vit. A deficiency. 149.
McCashland, B. W. 165.
McCluer, R. H. 216.
McCord, W. M., and Ezell, H. K. Blood, erythrocyte, volume, determination. 727.
McGinnis, J. 15, 233.
Meadows, G. B. 173.
Meisel, E. 523.
Meites, J., and Shay, J. C. Vit. B_{12} effects. 196.
Melampy, R. M., Cheng, D. W., and Northrop, L. C. Pantothenic acid, deficiency in mice. 24.
Mellette, H. C. 410.
Melnick, J. L. 312.
Melton, C. 361.
Menge, H., Moreng, R. E., and Combs, G. F. Nutrition, chick, yolk removal effect. 41.
Mersheimer, W. L., Kolarsick, A. J., and Kammandel, H. C. Ureter, implant, intestine. 170.
Meyer, D. K., and Westfall, B. A. Gill, fish, metabolism. 377.
Meyer, L. M., Brahin, C. M., and Sawitsky, A. Citrovorum factor, pernicious anemia. 86
Michael, M., Jr., and Whorton, C. M. Hormone, cortisone, inflammation. 754.
Mika, L. A. 786.
Milhorat, A. T. 408.
Militzer, W. 598.
Milman, A. E. 408.
Milnes, R. F. Vein, vena cava constriction, ascites. 147.
Milstone, J. H. Manganous ion and prothrombokinase. 33.
Moeller, H. C., Marshall, H. C., and Kirsner, J. B. Enzyme, lysozyme, intestinal injury. 159.
Moeller, H. C. 427.
Montagna, W. 35.
Montgomery, A. V. 773.
Mook, W. 403.
Moore, A. E. Virus encephalitis, oncolysis. 749.
Moreng, R. E. 46.
Morgan, C., and Mowry, R. W. Glycogen, demonstration, electron microscope. 850.
Morgan, J. F. 444.
Morton, H. J. 444.
Mosey, L., Maison, G. L., and Stutzman, J. W. Veratrum viride, after digitalis or quinidine. 486.
Mowry, R. W. 850.
Mulholland, J. H. 113.
Munoz, J. C. 384.

Nash, H. 480.
Necheles, H. 277.
Nedzel, G. A. 177.
Neidig, B. A. 783.
Nelson, A. A., and Lusky, L. M. Pyrrolidone, toxicity. 765.
Nelson, R. M., and Dennis, C. Bacillus, paracolon, bacteremia. 737.
Neter, E., Gorzynski, G. A., and Cass, W. A. Antibiotic, aureomycin, terramycin, neomycin, pasteurella. 493.
Neva, F. A. 560.
Newton, J. O. 65.
Newcomb, E. H. Auxin, ascorbic oxidase activity. 504.
Norman, M. C., Longfellow, D., and Levin, J. K. Antibiotic aureomycin, *L. monocytogenes*. 435.
Northrop, L. D. 24.
Northrup, M. 804.
Northup, D. W. 102.
Nungester, W. J., Bosch, J. K., and Alonso, D. Mucin, respiratory tract. 777.

Oda, J. M. 707.
O'Dell, B. L., Whitley, J. R., and Hogan, A. G. Vit. B_{12}, hydrocephalus prevention. 349.
O'Leary, J. F., Leary, D. E., and Slater, I. H. Pyridyl compounds, nervous system. 738.
Oleson, J. J. 676.
Orent-Keiles, E. 295.
Ottinger, B. 177.
Oyama, J. 177.

Pace, D. M., and McCashland, B. W. Cyanide, growth nad respiration effect. 165.
Palmer, D. 115.
Panos, T. C. 833.
Parker, R. C., Morgan, J. F., and Morton, H. J. Rubber, toxicity, tissue culture. 444.
Parsons, R. J. 831.
Paschkis, K. E. 432.
Peacock, W. M., Jr. 761.
Pearson, A. M. 173.
Pearson, H. E. 689.
Perera, G. A. Hormone, cortisone, blood pressure. 583.
Phillips, P. H. 642.

Pimenta de Mello, R. Coproporphyrin excretion, lead poisoning. 823.
Pinkerton, H. 475.
Plaut, G. W. E., and Smith, V. R. Acid caproic, metabolism. 104.
Plotz, C. M. 361.
Polley, J. R., Burr, M., and Gillen, A. Virus, influenza, antigens. 330.
Popper, H. L., and Necheles, H. Fat, necrosis, production. 277.
Port, S. 831.
Portwood, L. M. 307.
Potter, V. R. Sequential blocking, metabolic pathways, 38; Busch, H., and Bothwell, J. Metabolism tissue, *in vivo* studies. 41.
Powell, C. 477.
Pratt, A. W., and Kahler, H. Papilloma, electron microscopy. 656.
Proescher, F. Coagulant, anti, iminodiacetic acid. 619.
Pugh, B. L. 398.
Pulaski, E. J. 254.

Quastel, J. H. 183.
Quick, A. J., and Hussey, C. V. Blood, prothrombin, thromboplastin effect. 732.
Quinn, G. P. 601.

Raab, W., and Gigee, W. Brain encephalin, distribution. 97.
Radke, R. A. *Entamoeba histolytica*, cystic stage 831.
Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J. Isotope phosphorus, chlorouridine effect, neurotropic virus. 689.
Ramasarma, G. B. 714.
Ramenchik, A. 384.
Rasmussen, A. F., Jr. 659, 662.
Ray, C. T. 1.
Read, C. P. Tapeworm tissue, glycolysis. 861.
Reavis, J. C. 592.
Reiners, C. R., Jr. Bile, acids, jaundice. 757.
Reinertson, J. W., and Thompson, P. E. Liver, amoebiasis. 518.
Reinhardt, W. O., and Li, C. H. Hormone ACTH, antidiuretic activity. 836.
Revzin, A. M. 121.
Rhodes, A. J., and Clark, E. M. Virus poliomyelitis, antibody. 379.
Richardson, L. R., Witten, P. W., and Couch, J. R. Vit B_{12}, tissues. 265.
Richardson, L. R. Brain, hydrocephalus, nutritional. 142.
Richert, D. A., and Westerfeld, W. W. Enzyme, xanthine oxidases, species. 252.
Richmond, J. B. 384.
Rider, J. A., Moeller, H. C., and Dubois, K. P. Enzyme cholinesterase, method. 427.
Rigdon, R. H., and Breslin, D. Blood, erythrocytes, Heinz bodies. 242.
Riggs, H. E. 267.
Rinehart, J. F. 580.
Roberts, E., Karnofsky, D. A., and Frankel, S. Cortisone, hydroxyproline effect. 289.
Roberts, E. 714.
Roberts, J. M. 307.
Robinson, A. R. 704.
Rosenak, S. S., and Saltzman, A. Kidney, artifiscal. 471.
Rosenblum, C. 393.
Rosenkrantz, H., Milhorat, A. T., Farber, M., and Milman, A. E. Vitamin, antistiffness factor, identification. 408.
Rosenthal, M. C., and Schwartz, L. I. Erythrocyte, trypsin treated, agglutination. 635.
Rosenthal, T. B. 714.
Rothschild, L. 52.
Rubin, A., Horvath, S. M., and Mellette, H. C. Bacterial fecal, rectal temperature effect. 410.
Rubin, S. H. 18.
Rubin, S. J. 261.
Rubini, J. R., Rasmussen, A. F., Jr., and Stahmann, M. A. Polypeptides, lysine, influenza virus growth. 662.
Rubini, J. R., Stahmann, M. A., and Rasmussen, A. F., Jr. Blood, erythrocytes, agglutination polypeptides. 659.
Ruchman, I., and Fowler, J. C. Toxoplasma, persistence. 793.
Ruml, D., and Bohnhoff, M. Adrenal cortex, meningococcal infection. 679.
Rupp, J., Paschkis, K. E., and Cantarow, A. Vit. B_{12} nitrogen balance. 432.

Sabella, J. D., Bern, H. A., and Kahn, R. H. Vit. A, epidermis, 499.
Sackler, A. M. 226.
Sackler, M. D. 226.
Sackler, R. R., Sackler, M. D., Sackler, A. M., Greenberg, D., Van Ophuijsen, J. H. W., and Co Tui. Blood, eosinophiles, psychotics. 226.
Sacks, L. E., and Humphreys, E. M. Antibiotic, lupulone. 234.
Sakamoto, W., Gordon, G. S., and Eisenberg, E. Steroids, prolonged action. 406.
Sallman, B., Birkeland, J. M., and Gray, C. T. Enzyme hyaluronidase, streptococcal pathogenesis. 467.
Saltzman, A. 471.
Salvin, S. B. Fungi, hemolysin. 852.
Salzberg, H. 797.
Sarandria, J. L. 388.
Saunder, J. P., and Spaulding, R. C. ANTU, toxicity. 84.
Saunders, W. W. 592.
Sawitsky, A. 86.
Sayers, G. 65, 639.
Schafer, P. W. 375.
Schapira, R. 704.
Scharz, H. P., Riggs, H. E., Glick, C., Cameron, W., Beyer, E., Jaffe, B., and Trombetta, L. Insulin shock, infra red spectroscopy. 267.
Schlesinger, R. W. Virus, dengue, propagation. 817.
Schneider, B. H. 15.
Schrek, R. Hormone, adrenal cortex toxicity, test. 557.
Schwartz, I. L. 608, 612.
Schwartz, L. I. 635.
Scott, K. G., Reavis, J. C., Saunders, W. W., and White, W. E. Isotope iodine, thyroid function. 592.
Seaman, G. S. 169.
Seidlin, S. M., Yalow, A. A., and Siegel, E. Isotope iodine, blood, irradiation effect. 711.
Selkurt, E. E., and Glauser, K. F. Pentobarbital, sodium metabolism. 257.

Selye, H. Hormone DCA, somatotrophic toxicity. 510.

Shahinian, S. S. 441.

Shapiro, R., Taylor, B., and Taubenhaus, M. Hormone cortisone, granulation tissue. 854.

Shawver, C. B. 175.

Shay, J. C. 196.

Shorr, E. 446.

Sieburth, J. M. N., Gutierrez, J., McGinnis, J., Stern, J. R., and Schneider, B. H. Antibiotics, microflora, animal growth. 15.

Siegel, E. 711.

Silber, R. H. 395.

Silberberg, M., and Silberberg, R. Estrogen, tissue susceptibility. 161.

Silberberg, R. 161.

Silverman, F. N., and Mackler, B. Vitamin, folic acid, scurvy. 574.

Singer, L., and Armstrong, W. D. Isotope, calcium. 229.

Singer, M. Nerve, limb regeneration. 415.

Sjoerdsma, A. Digilanid, creatine reserve. 126.

Skelton, F. R. 77.

Skupp, C. 115.

Slater, I. H. 738.

Slater, R. J. 190.

Sloan, H. Tetrathionate, uremia production. 344.

Smith, M. W. 213.

Smith, V. R. 104.

Smith, W. M., Chambers, V. C., and Evans, C. A. Virus, influenza, growth, testicle. 696.

Smolelis, A. N., and Hartsell, S. E. Enzyme lysozyme, egg albumin. 455.

Soffer, L. J., Gabrilove, J. L., and Dorrance, W. R. Hormone, ACTH, thyroid iodine. 763.

Solotorovsky, M., Gregory, F. J., and Stoerk, H. C. Cortisone, vaccination. 286.

Southam, C. M., and Goldsmith, Y. Complement, nitrogen mustard. 430.

Spain, D. M., and Thalhimer, W. Blood eosinophile, spleen, cortisone. 320.

Spaulding, R. C. 84.

Stahmann, M. A. 659, 662.

Stanton, M. F. 475.

Stein, K. F., Martin, C., and Falk, D. Epinephrine, leucocyte count. 110.

Stern, J. R., and McGinnis, J. Vit. B_{12}, toxicity. 233.

Stern, J. R. 15.

Stickney, J. C. 102.

Stoerk, H. C. 286.

Stokstad, E. L. R. 806.

Stokstad, E. L. R., and Jukes, T. H. Vit. B_{12} requirement and aureomycin. 73.

Stolarsky, F. 299.

Stollerman, G. H., Rubin, S. J., and Plotz, C. M. Cortisone hypersentivity passive. 261.

Storey, W. H. 734.

Streicher, E. Colchicine, toxicity. 536.

Stulberg, C. S., Schapira, R., Robinson, A. R., Basinski, D. H., and Freund, H. A. Virus, influenza, mucoprotein effect. 704.

Stumpe, W. M. 825.

Stutzman, J. W. 486.

Sugg, J. Y. Virus influenza, mixture. 199.

Sussman, N. 683.

Swank, R. L., Franklin, A. E., and Quastel, J. H. Sclerosis, multiple, plasma chromatography. 183.

Swann, H. G., Montgomery, A. V., and Lowry, J. S. Kidney, vein occlusion. 773.

Swiss, E. D., and Bauer, R. O. Veratrum derivatives, toxicity. 847.

Tabenkin, B. 18.

Taubenhaus, M. 854.

Taylor, B. 854.

Taylor, D. J. 700.

Thalhimer, W. 320.

Thomas, L., and Good, R. A. Hormone cortisone, renal cortical necrosis. 604.

Thompson, P. E. 518.

Threefoot, S. A., Ray, C. T., and Burch, G. E. Isotope chloride 36 turnover. 1.

Timiras, P. S. 559.

Ting, K. S., and Coon, J. M. Procaine toxicity, hydrolytic products. 543.

Tocantins, L. M., Holburn, R. R., and Carroll, R. T. Blood, prothrombin, thromboplastin effect. 623.

Tolbert, B. M. 741.

Tonhazy, N. E. 546.

Travers, J. J., and Cerecedo, L. R. Methoxinine. 497.

Trombetta, L. 267.

Tucker, D. 361.

Tyler, A., and Rothschild, L. Spermatozoa, metabolism. 52.

Tyor, M. P. 250.

Ulrich, H. A., and Fitzpatrick, T. B. Vitamin, anti, neopyrithiamine, microsporum inhibition. 346.

Umbreit, W. W. 546.

Van Dyke, D. C. 707.

Van Dyke, H. B. 576.

Van Liere, E. J., Stickney, J. C., and Northup, D. W. Intestine motility, effect acclimatization. 102.

Van Noate, H. F. Heart auricle pressure, shock. 155.

Van Ophuijsen, J. H. 226.

Vaughan, J. H., Bayles, T. B., and Favour, C. B. Cortisone, blood protein effect. 274.

Villela, G. G., and Cury, A. Vit. biotin, assay. 341.

Visscher, M. B. 746.

Voorhees, A. B., Baker, H. J., and Pulaski, E. J. Dextran, metabolism. 254.

Wachstein, M., and Meisel, E. Acid, amino, mercurial toxicity. 523.

Wagner, J. C., Andrew, V. W., and Watson, M. S. Aureomycin, psittacosis. 79.

Wagner, J. W. 630.

Waisman, H. A., Green, M., Munoz, J. C., Ramenchik, A., and Richmond, J. B. Antibiotic aureomycin, citrovorum factor. 384.

Wang, S. C. 335.

Warren, S. 4, 709.

Watkins, F. B. 477.

Watson, J. 366.

Watson, M. S. 79.

Weintraub, S. 489.
Wenneker, A. S., and Sussman, N. Liver, regeneration, after hepatectomy. 683.
Wenner, H. A., Gibson, D. M., and Jaques, R. Enzyme hyaluronidase, streptococcal. 585.
Werner, C. A., Adams, C., and DuBois, R. Antibiotic, Viomycin, metabolism. 292.
Wertman, K., and Sarandria, J. L. Antibody, typhus, vitamin deficiency. 388.
Westerfeld, W. W. 252.
Westfall, B. A. 377.
Weston, R. E. 797.
Westphal, U., and Gedigk, P. Azorubin-binding, serum. 838.
White, A. 322.
White, W. E. 592.
Whitehair, C. K. 284.
Whitehill, A. L. 676.
Whitley, J. R. 349.
Whorton, C. M. 754.
Wiesel, L. L. 825.
Willcox, M. L. 3.
Williams, J. N., Jr., Feigelson, P., Shahinian, S. S., and Elvehjem, C. A. Nucleotide pyridine, formation. 441.
Williams, J. N., Jr. 828.
Wilson, H. W., and Wagner, J. W. Algin, feeding. 630.
Wilson, R. H., Booth, A. N., and De Eds, F. Flavonoids, histamine shock. 540.
Winzler, R. J. 689.
Wisseman, C. L., Jr. 533.
Wiswell, O. B. Arginase, tumor inhibition. 588.
Witten, P. W. 265.
Wolf, S. 398.
Wolfe, S. 366.
Wong, D. H., and Freund, J. Virus rabies, blood. 717.
Wood, H. F. Protein, C reactive, human leucocytes. 843.
Woodbury, D. M., Emmett, J. W., Hinckley, G. V., Jackson, N. R., Newton, J. O., Bateman, J. H., Goodman, L. S., and Sayers, G. Adrenal cortex brain excitability. 65.
Woodbury, D. M. 639.
Woodbury, D. T. 393.
Woods, M. C. 734.
Wright, L. T. 489.
Wright, M. R. Taste organ, maintenance. 462.
Wu, D. Y., and Wu, H. Saliva, urea. 130.
Wu, H. 130.
Wulff, V. J., Fry, W. J., Tucker, D., Frey, F. J., and Melton, C. Ultrasound, effect nerve. 361.

Yall, I. 152.
Yalow, A. A. 711.
Yamamoto, R. 393.

Zarrow, I. G. 620.
Zarrow, M. X., and Zarrow, I. G. Thiouracil, adrenal involution. 620.
Ziff, M. 649.
Zittle, C. A., and Dellamonica, E. S. Enzyme, phosphatase, alkaline, absorption. 193.
Zweifach, B. W., Black, M. M., and Shorr, E. Tetrazolium, adrenal cortex. 446.

SUBJECT INDEX

VOLUME 76

(*The numerals indicate the page*)

Prepared by Dr. William Antopol

Acetate sodium, distribution in egg. 741.
Acid, amino, dentin protein. 783.
  bile, in jaundice. 757.
  caproic, metabolism. 104.
  fatty, essential, metabolism. 100
  glycine, toxicity. 233.
  hydroxyproline, cortisone influence. 289.
  mercurial toxicity. 523.
  nucleic, glycine metabolism. 464.
    photodynamic action. 90.
  tryptophane, Borrelidin toxicity. 18.
  uric, diabetes. 642.
ACTH, Rickettsiae toxicity. 560.
  virus toxicity. 560.
Adenosine triphosphate, solubility. 247.
Algin, feeding. 630.
Alloxan, Bartonellosis. 475.
Altitude, consciousness time. 140.
Amoebae, hepatitis. 518.
Antibiotic aureomycin, alkali, activity. 676.
  B_{12} requirement. 73.
    citrovorum factor. 384.
    digestion. 284.
    L. monocytogenes. 435.
    pasteurella. 493.
    psittacosis. 79.
  Benemid. 222.
  Borrelidin, toxicity. 18.
  chloramphenicol, *L. monocytogenes*, 435.
  enzyme inhibition. 533.
  fowl nutrition. 58.
  fungicidin. 93.
  hexamitiasis. 522.
  Lupulone. 234.
  microflora, animal growth. 15.
  neomycin, pasteurella. 493.
  streptomycin toxicity. 466.
  terramycin, pasteurella. 493.
  synnematin. 307.
  tyrocidine, trypanosome. 744.
  viomycin, metabolism. 292.
Antibody, hemagglutinin, hetero. 210.
  pantothenic acid, methionine. 670.
    pyridoxine deficiency. 665.
  tissue, method. 460.
  tuberculin agglutinins, leprosy. 171.
  typhus, vit. deficiency. 388.
ANTU, toxicity. 84.
Arginase, tumor inhibition. 588.
Artery, elastin, amino acid. 714.
Auxin, ascorbic oxidase. 504.
Azorubin-binding, serum capacity. 838.

Bacillus, paracolon, bacteremia. 737.
Bacteria, Bartonella, alloxan. 475.
  Brucella, resistance and virulence. 786.
  fecal, rectal temperature. 410.
  Listeria monocytogenes, aureomycin, chloramphenicol. 435.
  pasteurella, antibiotic effect. 493.
  streptococci, hyaluronic acid. 467.
  thermophilic, cell granule. 598.
Benemid, antibiotic levels. 222.
Benzene hexachloride, metabolism. 780.
Blood, eosinophile, psychotics. 226.
    spleen, cortisone. 320.
  erythrocytes, agglutination, polypeptides. 659.
    Heinz bodies. 242.
    volume, ACTH effect. 707.
    determination. 727.
  factor O, newborn. 554.
  glutathione, cortisone, ACTH. 418.
  leucocyte, C reactive protein effect. 843.
    epinephrine. 110.
    riboflavin, pteroylglutamic acid. 804.
  pernicious anemia, citrovorum factor. 86, 366.
  potassium, newborn. 756.
  prothrombin, thromboplastin. 623, 732.
  sugar, adrenal function. 77.
  volume, method P^{32}. 831.
Bone, growth, cortisone. 722.
Brain, analysis, encephalomyelitis. 746.
  electrode, implant. 315.
  encephalin distribution. 97.
  excitability, adrenal cortex. 65.
  hydrocephalus, nutritional. 142.
Bromsulphthalein, excretion. 216.
Bufalin, digitalis-like effect. 372.

Calcium, determination. 115.
Cholate, sodium. 216.
Cholesterol, plasma, B_6 deficiency. 580.
Chondroitin sulfate, synthesis, cortisone. 596.
Cinobufagin, digitalis-like effect. 312.
Cinobufalin, digitalis-like effect. 372.
Citrate synthesis, X-ray. 422.
Citrovorum factor, pernicious anemia. 86, 366.
Coagulant anti, iminodiacetic acid. 619.
Colchicine, toxicity. 536.
  tumor growth. 105.
Collagen, metachromasia, proteolytic enzyme. 272.
Colon, DCA. 601.
Complement, nitrogen mustard. 430.
Consciousness time, altitude. 140.
Coproporphyrin excretion, lead poisoning. 823.
Creatine reserve, digilanid influence. 126.
Cyanide, growth and respiration effect. 165.
  toxicity. 536.

Dextran, blood, urine. 3.
  metabolism. 254.
Diabetes, uric acid. 642.
Diasone, toxoplasmosis. 548.
Digilanid, creatine reserve. 126.
Digitalis, toxicity, potassium metabolism. 797.
Digitoxin, effect. 250.
Dilantin, axon. 297.

Electrolyte metabolism, hypophysectomy. 608.
Entamoeba histolytica, cystic stage. 831.

- **Enzyme,** acetylcholinesterase, localization. 169.
  - ascorbic oxidase, auxin effect. 504.
  - cholinesterase, method. 427.
  - esterase, serum. 304.
  - glucuronidase, *E. coli.* 672.
    - serum. 304.
  - histamine anti-, plasma coagulation. 299.
  - hyaluronidase anti-, serum, cortisone, ACTH. 239.
    - variations. 238.
    - streptococcal pathogenesis. 467, 585.
  - inhibition, antibiotic effect. 533.
  - lysozyme, egg albumin. 455.
    - intestinal injury. 159.
  - oxidatives, formaldehyde. 133.
  - phosphatase, alkaline, adsorption. 193.
  - proteolytic, collagen metachromasia. 272.
  - pyrophosphatase, colchicine. 105.
  - sulfhydryl, furacin. 152.
  - xanthine oxidase, liver. 828.
    - species. 252.
- **Erythrocyte,** trypsin treated, agglutination. 635.
- **Eye,** abnormality, vit. E. 295.
  - vitreous, electron microscopy. 857.

- **Fat,** necrosis, production. 277.
- **Fluorophosphate,** toxicity. 536.
- **Fungi,** coccidioides, parasitic phase cultivation. 332.
  - cultivation, yolk sac. 258.
  - hemolysin. 852.
- **Furacin,** sulfhydryl, enzyme. 152.

- **Glutathione,** irradiation protection. 456.
  - protection radiation. 396.
- **Glycogen,** demonstration, electron microscope. 850.
- **Glycosides action,** emetic. 335.

- **Hair proliferation,** skin damage. 35.
  - squalene effect. 801.
- **Heart,** acceleration, chemoreceptors. 121.
  - auricle pressure, shock. 155.
  - coronary artery occlusion, shock. 134.
  - hypertrophy, KCl. 391.
  - mercury. 403.
- **Histamine,** anti, peritonitis. 496.
  - fungistosis of antihistaminics. 538.
  - gastrectomy. 375.
  - flavonoid effect. 540.
- **Hormone** ACTH, anatomic changes. 177.
    - antidiuretic activity. 836.
    - antihyaluronidase. 239.
    - blood glutathione. 418.
    - blood protein. 274.
    - brain. 353.
    - erythrocyte volume. 707.
    - hemoglobin spreading. 559.
    - pressor activity. 836.
    - serum phosphorus. 770.
    - sulfhydryl inhibition. 825.
    - thyroid iodine. 763.
  - adrenal cortex DCA antagonism. 65.
    - histochemistry. 446.
    - meningococcal infection. 679.
    - toxicity, test. 557.
    - function, blood sugar. 77.
    - involution, thiouracil effect. 620.
    - phosphocreatine, adenosine triphosphate. 546.
    - vit. C. 322.
  - androgen, estrogen antagonism. 811.
  - antidiuretic, inactivation. 576.
  - cortisone, anatomic changes. 177.
    - antihyaluronidase. 239.
    - blood glutathione. 418.
    - bone growth. 722.
    - blood pressure. 583.
    - blood protein. 274.
    - brain. 353.
    - chondroitin sulfate, synthesis. 596.
    - DCA antagonism. 65.
    - granulation tissue. 854.
    - hydroxyproline. 289.
    - hypersensitivity passive. 292.
    - inflammation. 754.
    - phosphocreatine, adenosine triphosphate. 546.
    - renal cortical necrosis. 604.
    - splenic eosinophilia. 320.
    - sulfhydryl inhibition. 825.
    - vaccination. 286.
    - virus. 116.
  - DCA, colon. 601.
    - antagonism. 65.
    - somatotrophic. 510.
  - estrogen, androgen antagonism. 811.
  - epinephrine, leucocyte count. 110.
  - estrogen, tissue susceptibility. 161.
  - gonadotropin, vit. A. 149.
  - growth, feather regeneration. 118.
    - hypoglycemic effect. 18.
    - water metabolism. 612.
  - methyl testosterone, prolonged action. 406.
  - prolactin, phosphorus metabolism. 639.
  - testosterone, anabolic effect. 748.
    - prolonged action. 406.
- **Hypersensitivity passive,** cortisone. 261.
- **Hypoglycemia,** growth hormone. 21.
- **Hypophysectomy,** electrolyte and water metabolism. 608.

- **Iguana,** biochemical studies. 175.
- **Insulin shock,** infra red spectroscopy. 267.
- **Inflammation,** cortisone. 754.
- **Intestine,** motility, acclimatization effect. 102.
- **Intocostrin,** toxicity. 536.
- **Isotope,** calcium, metabolism. 229.
  - chloride 36 turnover. 1.
  - cobalt, vit. B_{12}, metabolism. 393.
  - glycine, nucleic acid metabolism. 464.
  - gold, rat. 207.
  - macrophages. 480.
  - peritoneal fluid. 477.
  - Hafnium metabolism. 462.
  - I_{131} radioautograph. 4.
  - iodine, blood irradiation. 711.
    - thyroid. 592.
    - thyroid tumor production. 563.
  - phosphorus, chlorouridine effect, neurotropic virus. 689.
    - intestinal localization. 709.
    - rat. 207.
    - toxicity, vit. B_{12}, folic acid. 552.
  - P^{32} radioautograph. 4.

Kidney, artificial. 471.
cortical necrosis, cortisone. 604.
decapsulation, hypertension. 338.
hypertrophy, KCl. 391.
vein occlusion. 773.

Lead poisoning, coproporphyrin excretion. 823.
Leprosy, tuberculin agglutinins. 171.
Leukemia, spontaneous, transmission. 27.
Liver, amoebiasis. 518.
necrosis, yeast effect. 124.
regeneration, hepatectomy. 683.
Lobelin, tetraethyl ammonium. 725.
Longilobine, liver. 530.
Lymph, iron content. 221.
Lymphosarcoma, adrenal vit. C. 322.

Manganous ion, prothrombokinase. 33.
Melamine, tri-ethylene action. 489.
Mercury, heart. 403.
toxicity, amino acid. 523.
Mesantoin effect, axon. 297.
Metabolism, gill, fish. 377.
tissue, *in vivo* studies. 38.
sequential blocking. 41.
Methoxinine, effect. 497.
Microscopy electron, glycogen. 850.
papilloma. 656.
vitreous body. 857.
Mucin, respiratory tract. 777.
Mucus, gastric, acetylcholine stimulation. 578.
Muscle, chemistry, rheumatism. 649.
Myeloma, protein, gamma globulin. 190.

Nerve, deflection, experimental. 302.
limb regeneration. 413.
ultrasound effect. 361.
Ninhydrin, reduction, urine. 692.
Nitrate, depressor response. 759.
Nitrogen balance, vit. B_{12}. 432.
mustard, serum complement. 430.
Nucleoprotein, radiation. 646.
Nucleotide, pyridine, formation. 441.
Nutrition, chick, yolk removal effect. 46.

Ova, fertilization. 129.

Pamaquine, metabolite. 700.
Pancreas, duct, intubation. 113.
Papilloma, electron microscopy. 656.
Parabiosis intoxication. 833.
Pentobarbital, sodium metabolism. 257.
Peritoneum, pneumo, gastrectomy preparation. 484.
Phosphorus, metabolism, prolactin. 639.
Physiological recording. 761.
Polypeptides, lysine, erythrocyte agglutination. 659.
influenza, virus growth. 662.
Potassium chloride, cardio-renal hypertrophy. 391.
metabolism and digitalis toxicity. 797.
Procaine, antidotes. 62.
toxicity, hydrolytic products. 543.
Promin, toxoplasmosis. 548.
Protein, C reactive, human leucocytes. 843.
globulin gamma, myeloma protein. 190.
hemin chromoprotein, biosynthesis. 527.
iodinated, depletion. 371.
thermal coagulation. 10.
Prothrombokinase, manganous ion. 33.
Pyridyl compounds, nervous system. 738.
Pyrrolidone, toxicity. 765.

Radiation, citrate synthesis. 422.
endothelial damage. 734.
flavonoid effect. 282.
glutathione protection. 396, 456.
mouse susceptibility. 729.
nucleoprotein viscosity. 646.
Radioautograph, solubility studies. 4.
Rheumatism, muscle chemistry. 649.
Rubber, toxicity, tissue culture. 444.

Saliva, urea. 130.
Sclerosis, multiple, plasma chromatography. 183.
Shock, auricular pressure. 155.
avitaminosis C. 325.
coronary artery occlusion. 134.
Somatotrophin toxicity, DCA effect. 510.
Spectra, infra red, tissues. 267.
Spermatozoa, metabolism. 52.
Squalene, hair loss. 801.
Stomach, evacuation, thiourea. 788.
gastrin, vagus. 49.
mucus, neutralizing property. 398.
Succinate, tumor metabolism. 437.
Sulfhydryl, ACTH, cortisone. 825.

T-1824, extraction. 145.
Taste, phenyl thio-carbamide, genetics. 589.
organ, maintenance. 462.
Teeth, dentin protein, amino acid composition. 783.
Telocinobufagin, digitalis like effect. 372.
Temperature, rectal, fecal bacteria. 410.
Tetrathionate, uremia production. 344.
Tetrazolium, adrenal cortex. 446.
Thiouracil, adrenal involution. 620.
Thiourea, gastric evacuation. 788.
Thyroid, iodine, ACTH. 763.
tumor, after radioiodine. 563.
Thromboplastin, prothrombin. 623, 732.
Thyroxine, metabolites. 686.
Tissue, elastic, electron microscopy. 515.
Toxoplasmosis, diasone, promin effect. 548.
persistence. 793.
Trypanosome, tyrocidine effect. 744.
Tumor, lysis, Russian encephalitis. 749.
metabolism, succinate. 437.

Ultrasound, nerve. 361.
Urea, saliva. 130.
Ureter, intestinal implant. 170.
Urine, ninhydrin reducing. 692.

Vaccine BCG, viability. 311.
Vaccination, tuberculosis, cortisone. 286.
Vagus, gastrin. 49.
Vein, vena cava constriction, ascites. 147.
Veratrum derivatives, toxicity. 847.
viride, after digitalis or guanidine. 486.
toxicity. 767.
Viritis, cortisone. 116.
Virus Col-MM, hemagglutination. 68.
Coxsackie, oral administration. 312.

  dengue, propagation. 817.
  encephalitis Japanese B. 566.
    oncolysis. 749.
  encephalomyelitis, brain analysis. 746.
  influenza, antigen. 330.
    growth, in testicle. 696.
    inapparent infection. 570.
    lysine polypeptide effect. 662.
    mixture. 199.
    mucoprotein effect. 704.
  mumps, distribution in monkey. 571.
  neurotropic, testis. 213.
    phosphorus metabolism. 689.
  poliomyelitis, antibody. 379.
    intraspinal inoculation. 357.
  psittacosis, aureomycin. 79.
  rabies, blood. 717.
Vitamin A, deficiency, gonadotropin. 149.
    epidermis. 499.
  anti-methopterin, citrovorum factor antagonism. 382.
    neopyrithiamine, microsporum inhibition. 346.
    stiffness factor, identification. 408.
  APF, multiple factors. 173.
  B_6, plasma cholesterol. 580.
  B_{12}, assay. 806.
    effects. 196.
    folic acid, P^{32} toxicity. 552.
    hydrocephalus prevention. 349.
    metabolism. 393, 720.
    nitrogen balance. 432.
    requirement, aureomycin. 73.
    tissue content. 265.
    P^{32} toxicity. 552.
    toxicity. 233.
  biotin, assay. 341.
  C, adrenal. 322.
    shock. 325.
  citrovorum factor, aureomycin. 384.
    methopterin competition. 382.
    oviduct stilbestrol response. 203.
    pernicious anemia. 86, 366.
  deficiencies, typhus antibodies. 388.
  E, eye abnormality. 295.
  folic acid, scurvy. 574.
  flavonoids, histamine shock. 540.
    radiation. 282.
  folinic acid, pteroylglutamic acid replacement. 417.
  niacin, Borrelidin toxicity. 18.
  pantothenic acid, deficiency, antibodies. 665.
    deficiency. 24.
    methionine effect. 670.
  pteroylglutamic acid, leucocyte production. 804.
    replacement folinic acid. 417.
  pyridoxine deficiency, antibodies. 665.
  riboflavin, leucocyte production. 804.

Water metabolism, growth hormone effect. 612.
  hypophysectomy. 608.

(Continued from page xvi)

SCHLESINGER, R. W.	Propagation in Chick Embryos of Dengue Virus, Hawaiian Strain. Findings in Infected Eggs	817
SCHWARTZ, L. I., 635.		
SEIDLIN, S. M., YALOW, A. A., and SIEGEL, E.	Irradiation Produced Rise in Blood Radioiodine Concentration Following Ingested Therapeutic Dose for Metastatic Thyroid Carcinoma	711
SHAPIRO, R., TAYLOR, B., and TAUBENHAUS, M.	Local Effects of Cortisone on Granulation Tissue and the Role of Denervation and Ischemia	854
SIEGEL, E., 711.		
SLATER, I. H., 738.		
SMITH, W. M., CHAMBERS, V. C., and EVANS, C. A.	Growth of Neurotropic Viruses in Extraneural Tissues. Poliomyelitis Virus in Human Testicular Tissue *in vitro*	696
SOFFER, L. J., GABRILOVE, J. L., and DORRANCE, W. R.	Effect of Adrenocorticotropin on Thyroidal Collection of I_{131} in the Adrenalectomized and Intact Rat	763
STAHMANN, M. A., 659, 662.		
STOKSTAD, E. L. R., 806.		
STOREY, W. H., 734.		
STULBERG, C. S., SCHAPIRA, R., ROBINSON, A. R., BASINSKI, D. H., and FREUND, H. A.	Inhibition of Influenza Virus Hemagglutination by Purified Plasma Mucoproteins	704
STUMPE, W. M., 825.		
SUSSMAN, N., 683.		
SWANN, H. G., MONTGOMERY, A. V., and LOWRY, J. S.	Effect of Renal Venous Occlusion on Intrarenal Pressure	773
SWISS, E. D., and BAUER, R. O.	Acute Toxicity of Veratrum Derivatives	847
TAUBENHAUS, M., 854.		
TAYLOR, B., 854.		
TAYLOR, D. JANE, 700.		
TOCANTINS, L. M., HOLBURN, R. R., and CARROLL, R. T.	Response of Plasma to Excess of Thromboplastin as Measure of Prothrombin Activity	623
TOLBERT, B. M., 741.		
VAN DYKE, D. C., 707.		
VISSCHER, M. B., 746.		
WAGNER, J. W., 630.		
WARREN, S., 709.		
WENNEKER, A. S., and SUSSMAN, N.	Regeneration of Liver Tissue Following Partial Hepatectomy in Parabiotic Rats	683
WESTON, R. E., 797.		
WESTPHAL, U., and GEDIGK, P.	Azorubin-Binding Capacity of Normal and Pathological Sera	838
WHITEHILL, A. L., 676.		
WHORTON, C. M., 754.		
WIESEL, L. L., 825.		
WILLIAMS, J. N., JR., 828.		
WINZLER, R. J., 689.		
WONG, D. H., and FREUND, J.	Fixed Rabies Virus in Blood Following Intracerebral Inoculation in Mice and Rabbits	717
WOOD, H. F.	Effect of C-Reactive Protein on Normal Human Leukocytes	843
WOODBURY, D. M., 639.		
WOODS, M. C., 734.		
YALOW, A. A., 711.		
ZARROW, I. G., 620.		
ZARROW, M. X., and ZARROW, I. G.	Mechanism of Adrenal Involution of the Rat after Treatment with Thiouracil	620
ZIFF, M., 649.		